FIELD-PROGRAMMABLE ASSEMBLIES OF ARTIFICIAL CELLS AS INSTANCES OF A NEW EMBEDDED LIVING INFORMATION COMMUNICATION TECHNOLOGY

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CONTENTS

SUMMARY

As part of the efforts of the European Commission and Swiss National Science Foundation to generate a new information communication technology (ICT) by means of living technology (LT), the core aspect addressed in this thesis was the fabrication of a prototype of a field-programmable assembly of artificial cells (FPAAC), as an instance of a new embedded living information communication technology (LICT).

The established fields of nanotechnology and synthetic biology, as well as the only recently proposed area of LT that intends to create artificial cells from scratch, will characterize the scientific research and technological development of the 21st century, because they all focus on increasing the adaptivity, robustness, and versatility of man-made technology. Nanotechnology and synthetic biology both miniaturize concepts of 'classical' macroscopic engineering to the molecular scale either by designing and building machines atom by atom or by reprogramming existing organisms using artificial genetic building blocks. In contrast, LT conceptually spans a wider range because it incorporates biological concepts like emergence, self-organization, and the exploitation of the physico-chemical properties inherent to natural substances.

The natural sciences and ICT each accentuate the significance of 'embodiment' and hence the exploitation of material properties. To reduce either the genetic load or the computational effort, the exploitation of inherent material properties was identified as the core characteristic of adaptive, robust, and versatile natural or artificial systems. Ever since customizable and evolvable hardware became available in the 1990s, in the form of the field-programmable gate array (FPGA), the exploitation of inherent material properties was renowned in ICT. The lack of a field-programmable artificial cell (FPAC) resulted in research efforts being initiated by the European Commission and the Swiss National Science Foundation.

This thesis contributed its mite to shift the notion of programmability, information processing, and functionality in the new embedded LICT. The two main issues addressed in this thesis are as follows: Which components and which morphology allow for programmability, information processing, and functionality? Which functionalities are conceivable for FPAACs?

In addition to single-molecule components that autonomously arrange themselves into useful conformations, i.e., membrane-enclosed volumes as preliminary stages of artificial cells, this thesis employed a second level of supramolecular chemistry to unveil the potential offered by self-assembled artificial cells. Because the FPAACs are materially based on nature, one has to deal with a wealth of molecules from the very outset whose properties and myriad interactions define the material they compose. One can, therefore, neither introduce the material properties one by one as in embedded ICT, nor abstract from the molecular level as often seen in simulations.

The thesis pointed out the importance of assembled artificial cells that constitute a network of programmable composition and spatial arrangement. Moreover, the new laboratory protocols that were developed increase vesicle handling and manageability, and new preparation techniques internally define and structure the vesicle lumen. The vision of LT to invent life a second time, and to create a qualitative jump as man-made technology itself becomes alive, is ambitious and may take time. Therefore, scenarios are provided herein regarding how preliminary states of LT that are not alive but feasible may be used in personalized healthcare and ICT. The insights of this thesis heavily influenced the scientific direction of completed and current international research projects in this field.

ZUSAMMENFASSUNG

Als Teil der Bemühungen der Europäischen Kommission und des Schweizerischen Nationalfonds zur Schaffung einer neuen Informations- und Kommunikationstechnologie (engl. information communication technology, ICT) mittels belebter Technologie (engl. Living Technology, LT) hat sich diese Doktorarbeit der Herstellung eines Prototypen eines durch den Anwender programmierbaren Zusammenschliessens künstlicher Zellen (engl. field-programmable assembly of artificial cells, FPAAC) gewidmet. Dieser Prototyp sollte dabei als Beispiel einer neuen eingebetteten und belebten Informations- und Kommunikationstechnologie (engl. living information communication technology, LICT) dienen.

Sowohl die etablierten Forschungsgebiete der Nanotechnologie und der synthetischen Biologie, als auch das erst kürzlich vorgestellte Feld der belebten Technologie, die künstliche Zellen von Grund auf erschaffen will, werden die wissenschaftliche Forschung und die technologischen Entwicklungen des 21. Jahrhunderts prägen, da alle Richtungen ihr Hauptaugenmerk auf eine Erhöhung der Adaptivität, Robustheit und Vielseitigkeit menschgemachter Technologie legen. Sowohl Nanotechnologie als auch die synthetische Biologie miniaturisieren dabei Konzepte der ‚klassischen' makroskopischen Konstruktionsweise und wenden diese auf die molekulare Grössenordnung an. Dadurch werden entweder Maschinen Atom für Atom konzipiert und gebaut oder es werden existierende Organismen unter Verwendung künstlicher genetischer Bausteine umprogrammiert. Indem Konzepte wie Emergenz, Selbstorganisation und die Nutzung der physikalisch-chemischen Eigenschaften natürlicher Substanzen eingebunden werden, deckt die belebte Technologie im Gegensatz zur Nanotechnologie und der synthetische Biologie einen grösseren Bereich ab.

Ergebnisse der Naturwissenschaften und der Informations- und Kommunikationstechnologie heben die Bedeutung der Körperlichkeit (engl. embodiment) und damit die Nutzung von Materialeigenschaften hervor. Die Nutzung inhärenter Materialeigenschaften wurde als Kernstück adaptiver, robuster und vielseitiger künstlicher Systeme ausgewiesen; damit kann sowohl die genetische Last als auch der Berechnungsaufwand reduziert werden. Seit anpassbare und evolvierbare Hardware in den 1990ern in der Form anwenderprogrammierbarer Logikschaltungen (engl. field-programmable gate array, FPGA) zugänglich wurden, wurde die Bedeutung der Nutzung inhärenter Materialeigenschaften in der Informations- und Kommunikationstechnologie erkannt. Das Fehlen anwenderprogrammierbarer künstlicher Zellen (engl. field-programmable artificial cell, FPAC) hat die Europäische Kommission und den Schweizerischen Nationalfonds zu Forschungsanstrengungen in dieser Richtung bewogen.

Diese Doktorarbeit hat ihren Teil dazu beigetragen die Wahrnehmung von Programmierbarkeit, Informationsverarbeitung und Funktionalität in einer neuen eingebetteten belebten Informations- und Kommunikationstechnologie zu verändern. Die zwei Schwerpunkte dieser Doktorarbeit waren dabei: Welche Komponenten und welche Ausgestaltung erlauben Programmierbarkeit, Informationsverarbeitung und Funktionalität? Welche Funktionalitäten sind vorstellbar für durch den Anwender programmierbare Zusammenschlüsse künstlicher Zellen?

Zusätzlich zu den Einzelmolekülkomponenten die sich autonom zu günstigen Ausformungen organisieren, beispielsweise als membranumschlossene Volumen, die als Vorstufen künstlicher Zellen angesehen werden können, hat diese Doktorarbeit eine zweite Stufe supramolekularer Chemie herangezogen um das Potenzial selbstorganisierter künstlicher Zellen zu enthüllen. Weil die künstlichen Zellen, die sich durch den Anwender programmiert zusammenschliessen, im Wesentlichen auf der Natur beruhen, muss man von Anfang an mit einer Fülle von Molekülen umgehen, deren Eigenschaften und vielfältige Interaktionen die Materialien bestimmen, die sie aufbauen. Aus diesem Grund können weder die Materialeigenschaften nacheinander eingeführt werden, wie dies bei der Informations- und Kommunikationstechnologie möglich ist, noch kann von der molekularen Ebene abstrahiert werden, wie dies in Simulationen oft geschieht.

Die Doktorarbeit hat die Bedeutung zusammengeschlossener künstlicher Zellen, die ein Netzwerk von programmierbarer Zusammensetzung und räumlicher Anordnung bilden, hervorgehoben. Des Weiteren wurden nebst neuen Laborprotokollen, welche die Handhabung und die Verwaltung künstlicher Zellen erhöhen, neue Verfahren zur internen Strukturierung des Lumens künstlicher Zellen entwickelt. Die Vision belebter Technologie das Leben ein zweites Mal zu erfinden und einen qualitativen Technologiesprung zu bewirken, wenn menschgemachte Technologien zu leben beginnen, ist ambitioniert und braucht vermutlich noch etwas Zeit. Aus diesem Grund wurden Szenarien entwickelt wie Vorstufen belebter Technologie, die selbst zwar noch nicht lebendig aber technisch möglich sind, in personalisierter Gesundheitsfürsorge und in der Informations- und Kommunikationstechnologie eingesetzt werden können. Die Einsichten, die diese Arbeit mit sich brachte, haben die wissenschaftliche Ausrichtung abgeschlossener und aktueller internationaler Forschungsprojekte in diesem Gebiet massgeblich beeinflusst.

CHAPTER 1

'FIELD-PROGRAMMABLE ASSEMBLIES OF ARTIFICIAL CELLS': HOW 'INFORMATION COMMUNICATION TECHNOLOGY' AND 'LIVING TECHNOLOGY' GIVE RISE TO 'LIVING ICT'

1 Living Information Communication Technology

As part of the PACE[1] and EES[2] projects, the core aspect addressed in this thesis was the fabrication of prototypes of field-programmable assemblies of artificial cells (FPAAC) as an instance of a new embedded living information communication technology (LICT).

The current state of LICT is comparable to the situation of information communication technology (ICT) 20 years ago. Ever since field-programmable gate arrays (FPGA) became available in the 1990s, the exploitation of material properties became resound in ICT. FPAACs may reshape our notion of computation like the evolvable hardware FPGAs shaped the way people think of intelligence.

1.1 ICT and LT: Similar in Concept…

1.1.1 Concepts in ICT

Researchers in artificial life (AL) and artificial intelligence (AI) increasingly agree that the core features of life, such as robustness, autonomy, self-repair, adaptation, and self-replication, can strongly inform the design of ICT to create man-made systems that become alive and/or intelligent.

The use of computers to evolve solutions to problems *in silico* strongly influenced research in AL. Based on the early Mendelian notion of 'one gene, one function' dating back to the 'one gene, one enzyme' hypothesis of Beadle and Tatum [1], the first evolutionary algorithms in AL focused on one-to-one mapping from genotype to phenotype. Even though this simplification was identified to be inappropriate, the perception of gene action, i.e., one gene codes for one phenotypic trait, is still prevalent among lay people and had a long shelf life in AL (for a discussion of the misconceptions resulting from the early Mendelian view see [2]).

Today, more plausible biological perceptions of gene action are widely accepted in AL and constitute the research field of 'embryogenic evolution' also called 'computational evolution', [3-5]. This shift in paradigm was initiated by the work of Cangelosi and Nolfi [6], Eggenberger Hotz [7], and Bongard [8]. Bentley and Kumar [9] discussed inherent scalability of natural embryogeny, indirect mapping of genotypes to phenotypes, and mimicking of developmental processes. Like nature, these new evolutionary algorithms make use of the unimaginable convolutions of interaction between all parts of a growing creature, permit a small amount of information to generate immensely more complex forms, and implicitly encompass concepts such as compression, iteration, recursion, adaptation, and memory. Due to its scalability, once evolved in nature, this basic concept allowed for the evolution of every creature, ranging from bacteria to human.

In addition to developmental processes, Eggenberger Hotz [10] was able to further increase the robustness and evolvability by introducing simulated physics to evolutionary algorithms. Banzhaf [3] subsequently resumed this approach and stressed that the incorporation of physics is one of the main points on the AL research agenda to develop the new field of computational evolution that *"could solve previously unimaginable or intractable computational and biological problems."*

Prior to the seminal influence on AL, the significance of physics was acknowledged in AI. Brooks [11], Pfeifer [12], and Steels [13] were pioneers in arguing that true artificial intelligence can only be achieved by machines that are connected to the world through a body. This embodied intelligence finally resulted in the concept of 'morphological computation' recently coined by Paul [14]. Beforehand, AI was largely about neural modeling (e.g., phonotaxis, navigation, and vision) restricted to higher level cognition processes, and focused on a highly elaborated brain without a body. This top-heavy concept contrasts natural systems, which are the product of a complex, everlasting, reciprocal, and dynamical coupling among the brain, the body, and the environment. In his recent review [15], Pfeifer lists the following principles that trace back to work done by himself, Collins, Dickinson, and Lun-

[1] **PACE**: **P**rogrammable **A**rtificial **C**ell **E**volution. European Commission, 6th Framework Programme, IST-2002-2.3.4.2 FET pro-actives Integrated Project: 04/04-06/08.

[2] **EES**: **E**mbryogenic **E**volutionary **S**ystem: From Simulations to Robotic Applications. Swiss National Foundation 200020-118127: 10/05-09/09.

garella and that are implicitly or explicitly employed by robot designers: (i) the behavior of any system is not merely the outcome of an internal control structure but is also affected by the ecological niche in which the system is physically embedded, its morphology, and the material properties of the elements composing the morphology [16]; (ii) physical constraints shape the dynamics of the interaction of the embodied system with its environment [17, 18]; (iii) internal information processing is enhanced because embodiment and information are directly linked; coupled sensory-motor activity interacting with body morphology induce statistical regularities in sensory input and the control architecture [19]; and (iv) the notion of the embodied agent as complex dynamical systems enables us to employ concepts such as self-organization and emergence rather than hierarchical top-down control.

By putting physical aspects of morphology and material properties, as well as concepts like emergence and self-organization, in the spotlight of ICT, some key ideas of biology are resumed. In 1941, Thompson argued that the same mathematical concepts govern both the living and non-living world [20]. His considerations and conclusions were derived from a reflection on the mathematical and physical rules governing nature. He exemplified his concept by a vast number of analogies such as the Young-Laplace equation that characterizes the minimal surfaces of aggregated soap bubbles and the morphology of dividing cells. He argued that structures like helices, spirals, hexagons, cones, and fractals obviously are clearly an advantage for living beings, because they represent a splendid means for optimization and for achieving the most efficient structures in the most economic way.

However, biological research only recently started to explicitly analyze processes that integrate physical properties and information of different types and from different sources to provide specificity to morphogenesis. Keller *et al.* recently summarized this idea, writing [21]: *"The outcome of a local cellular* […] *event depends not only on its intrinsic properties, but its physical context, specifically the geometry and mechanical properties of adjacent cells or tissues* […]*."*

Insights from the natural sciences hence teach us that life codes for emergence, self-organization, and exploitation of the physico-chemical and morphological properties inherent to natural systems. When applied to man-made technology, these concepts may increase the adaptivity, robustness, and versatility.

1.1.2 Concepts in LT

The robustness, autonomy, self-repair, adaptation, and self-replication not only gave direction to ICT, but also to living technology (LT), which aims *"to capture the technological implications of our increasing ability to engineer systems whose power is based on the core features of life"* [22]. LT researchers consider it their scientific mission using a bottom-up approach to create minimal living units that will induce a qualitative jump as man-made technology itself becomes alive.

In contrast to ICT, LT is not only conceptually but also materially based on nature. Consequently, the term 'wetware' is introduced in LT (cf., 'software' and 'hardware' in ICT), pointing out that real-world experiments are performed in wet laboratory settings using organic and inorganic molecules. Certain forms of LT have existed for some time in research in the origin-of-life [23], synthetic biology [24], and astrobiology [25]. However, the identification and recognition of the concept is quite recent and gave rise to the European Center for Living Technology (Venice, Italy, founded in 2004) and the Center for Fundamental Living Technology (Odense, University of Southern Denmark, founded in 2007). Furthermore, LT was central to a variety of European Commission, Swiss National Science Foundation, and Los Alamos National Laboratory grants in 2004, 2005, 2007, 2008, and 2009.

In his seminal lecture "There's Plenty of Room at the Bottom" given at an American Physical Society meeting at Caltech on December 29, 1959 (for a transcript see [26]), Feynman defined nanotechnology (although not using this term) as building machines at the molecular scale atom by atom. Nowadays, the term is used in a wider sense and conveys all studies of controlling of matter on an atomic and molecular scale. Even though this thesis will show that the concept of building machines atom by

atom diametrically opposes the concepts of LT, LT aims to control nanoscopic materials (phospholipids, proteins, nucleotides etc.) at a molecular level and may thus be seen as an instance of nanotechnology.

1.2 ICT and LT: … but Distinct in Challenges

Although emergence, self-organization, and exploitation of the physical and morphological properties of materials are primary ingredients of LT and robotics in AI, they differ in the challenges they face. By choosing 'proper' materials to fabricate components of the body of a robot (e.g., inelastic limbs coupled by joints of defined degrees of freedom), robot designers in AI implicitly exploited morphological computation, i.e., to specify and to control the spatial pose of a linkage, the inelasticity of the limbs reduces the computational effort compared to elastic limbs. In this former example, a reduction in material complexity is beneficial. In other examples, however, an increase in complexity may be beneficial to the computational effort (see [15] for comprehensive list). Hence, one of the most important achievements of morphological computation is spelling out the explicit selection of 'proper' material properties in robot design.

Whereas ICT and LT are based on similar concepts, the challenges faced in ICT and LT fundamentally differ. Starting from elaborated algorithms, AL and AI only conservatively incorporated ideas of outsourcing functionality to inherent material properties to reduce the computational effort and to increase robustness of the technology. In AI robotics, the outsourcing is characterized by a gradual introduction of new dynamics provided by materials and morphological properties; influences on the controller and the performance of the system can be quantified iteratively. In AL, even if physics is introduced, simulations generally abstract from molecular details.

Because LT is materially based on nature, it has to deal with a wealth of molecules from the very outset whose properties and myriad interactions define the material they compose. One can, therefore, neither introduce new material properties one by one nor abstract from the molecular level; i.e., one cannot treat assemblies of lipid molecules as though they were 'just' a membrane of defined properties – molecular properties (mobility of single phospholipids etc.) are as important as supramolecular ones (self-repair characteristics etc.). The material properties, physical constraints, and concepts like self-organization and emergence have to be considered from the very outset. Compared to ICT, the challenges in LT are, therefore, exactly opposite; one does not have to find components that simplify control but ways to control a myriad of material properties inevitably inherent to the components. As a consequence, LT has to choose the components and morphology of their assemblies according to the programmability, information processing capacities, and the functionality provided by the system in its entirety.

2 ICT-LT projects

Concepts like programmability, information processing, and functionality brings ICT to the scene of LT. The PACE and EES projects were explicitly set up to bring together various insights of natural sciences, ICT, and LT, as well as to cope with the new challenges they impose. This thesis was funded by EES. Due to the overlap in motivation, theoretical and practical background, and research focus of EES and PACE, both projects are presented below.

2.1 Embryogenic Evolutionary System

The genome does not code for a one-to-one mapping from genotype to phenotype, but records how life controls and exploits the physico-chemical properties inherent to natural substances. As a result, the EES project promoted the encoding of self-organization, emergence, and the exploitation of material properties. Due to the inherent scalability of the natural model, it was intended that a small amount of information generates immensely more complex morphologies and features, while increasing the adaptivity, robustness, and versatility of the system. The scalability was tested by evolving controllers of robotic systems as diverse as macroscopic industrial manipulators, highly non-linear artificial muscles, and modular robots, in addition to a wet-

ware system composed of microscopic artificial vesicles detailed in this thesis. To cover this range, the EES project was split into a macroscopic ICT and a nanoscopic LICT branch. The macroscopic ICT branch was handled by Gómez and the nanoscopic LICT branch gave rise to this thesis.

2.1.1 Macroscopic ICT Branch of EES

In current robotic systems, the set of sensor positioning and movements allowed is strictly limited. Because changes in receptor or morphology layout offer the possibility of customizing robotic platforms, a neural controller must be able to reconfigure itself to cope with these changes. This would be a complex problem to solve for a designer because all aspects would need to be covered. A system able to explore its own movements and to coherently adapt its own behavior and morphology to the new situation is a more promising strategy.

The biologically inspired controllers, which were based on embryogenic, chemical, and mechanical mechanisms and which were implemented and evaluated *in silico* by Eggenberger Hotz in the scope of the HYDRA[3] project [27], were applied to conventional industrial robot platforms, to actuators (using highly non-linear artificial muscles), and to modular robots. Gómez demonstrated that these controllers outperform current strategies in both evolving different aspects of robotic systems (shape, sensory distribution, motor positions, neural structure, learning mechanisms, etc.) and in solving specific tasks such as reaching, grasping and tracking [28-30].

2.2 Programmable Artificial Cell Evolution

The integrated PACE project focused on fully embodied primitive life-like self-organizing artificial cells in a bottom-up strategy. Because LT in general aims to invent life for the second time [31, 32], abiogenesis, i.e., the spontaneous generation of life from inanimate matter, is one of its central aspects. Even though this question traces back to Aristotle [33], the PACE project represents the first concrete step towards artificial man-made life [34]. The name of the project already calls attention to its main aspects, i.e., artificial cells, programmability, and evolution.

Living artificial cells were intended to be realized in a bottom-up strategy from non-living source materials by implementing metabolic processes, inheritance, and information processing that induce viability, division, and information transfer over generations of artificial cells. The consortium intended to implement single compartments that converged towards living cells.

To reduce the complexity of the problem, aspects featuring natural living systems should have been first realized extrinsically on a microfluidic system and then gradually become intrinsic to the artificial cells. For instance, instead of producing energy to power their metabolism on their own, compartments would have been circling around in the microfluidic channels and been recharged by induced fusion with smaller ATP-loaded compartments. The energy production would have subsequently become intrinsic as the metabolism produced its own ATP [32]. Hence, 'programmability' was mainly set up to stress the precise control and manipulation of fluids using electronic microfluidic technology, i.e., to program the whereabouts of the artificial cells.

Although swarm intelligence, artificial immune networks, and agent-based modeling have produced promising results, imitating natural evolution is still the most popular method to solve optimization and design problems in AL [35]. Analogously, the design and complexity of the artificial cells should have been increased by drawing on evolutionary processes. Tracing back to the seminal work of Szostak *et al.* [36], the variance in the setting of different artificial cell populations should have caused differences in survival, i.e., differences in cell viability in dependence of external or internal factors. Like the Braitenberg vehicles that fall off the table [37], instances of the cell populations that did not survive, should have been replaced by new settings arising from mutations and recombinations in a Darwinian evolution.

[3] **HYDRA**: "Living" Building Blocks for Self-designing Artefacts. European Commission, 5th Framework Programme, IST-2001-33060 FET open domain, duration: 11/01-10/04.

Origin of life research focuses on the synthesis of life using basic non-living components to create living 'protocells' (see [38] for a recent review). The LT aspect of PACE was covered by the implementation of artificial cells following the same objective as the protocell research. Unique to the PACE project was its ICT aspect, which was characterized by (i) evaluating the potential and necessity of artificial cells for evolvable and scalable ICT, (ii) developing the capability to evolutionarily program artificial cells, (iii) determining the programmable potential of multi-cellular artificial systems, (iv) complementing, evolving, programming, and evaluating artificial cell functions, and (v) evaluating the potential application areas of this technology. The Artificial Intelligence Laboratory of the University of Zurich (AI Lab) was engaged in all ICT aspects except the simulation work.

3 Motivation of the Thesis

"Those who cannot remember the past are condemned to repeat it." This quote of Santayana [39] eloquently illustrates the motivation of this thesis. Even though the paradigm shift from the implicit to explicit exploitation of morphology and material properties took place in AL and AI a few decades ago, the new research field of LT only marginally employs these insights. This shortcoming was even represented in the sketch of PACE. 'Programmability' was mainly attributed to the precise control and manipulation of fluids in microfluidic channels, instead of referring to the programmability of matter by exploiting inherent material properties. Moreover, the 'Artificial Cells' were stipulated to be single, individual, and not divided into sections. In doing so, most features of morphology of natural organisms that are composed of several, assembled, and subdivided cells were completely disregarded.

From the five ICT aspects of PACE mentioned above, three central issues were formulated to outline this thesis as part of the AI Lab's effort to link ICT and LT, namely (a) which components and (b) which morphology allow for programmability, information processing, and functionality?; (c) which functionalities are conceivable for FPAACs?

To investigate these issues a basic wetware system was fabricated as a prototype of a FPAAC. Based on the insights gained using this wetware system, sound scenarios were elaborated on how to generate a new LICT that uses programmable wetware systems.

4 Significance of the Thesis

This thesis significantly contributed to link ICT and LT. The first implementation of multi-vesicle assemblies of programmable architectures represents crucial progress towards field-programmable artificial cells (FPAC), which exploit the material and morphological properties of the components to provide programmability, information processing, and functionality and thus a new LICT. Moreover, novel experimental protocols were established that allow for large scale production of artificial vesicles, increase procedural manageability, allow for high-throughput analyses of parameters affecting artificial vesicles, and enable to internally structure vesicles by artificial organelles. The potential of the novel technology was assessed by outlining how chemistry could be controlled and programmed in space and time. By pointing out the importance of the material and morphological properties, this thesis heavily contributed either to change or even set the course of completed (EES; PACE, [40]), current (MATCHIT[4], [41]), and future LT research and may reshape our notion of computation.

5 Thesis outline

The choices of components and morphology made at the beginning of the thesis are presented in Chapter 2. Chapters 3 to 5 are dedicated to the programmability of matter and morphology. Chapter 6 addresses the information processing capacities offered by the components and the morphology. Chapters 7, 8 and appendix 1 deal with the functionality of the system by providing scenarios of how this new technology could be applied to bio-

[4] **MATCHIT**: **MAT**rix for **CH**emical **I**nformation **T**echnology. European Commission, 7th Framework Programme, ICT-2009.8.3 FET Proactive 3: 02/10-01/13.

reactors, personalized healthcare, software-wetware testbeds, and the design of novel antiviral drugs. The concluding discussion in Chapter 9 comments on how the components and the morphology of the wetware system provide programmability, information processing, and functionality.

Chapters 3 to 5, 7, 8 represent manuscripts either prepared to be published (chapter 3) or published in the open-access journal PLoS ONE (chapter 4, [42]) and by Springer in the 'Lecture Notes in Computer Science' (chapters 5, 7, 8 [43-45]), respectively. Talks concerning the subject matters of chapters 5, 7, 8 were given either at the '4th Australian Conference on Artificial Life' (ACAL'09, University of Melbourne, Australia, 1-4 December, 2009; chapters 5, 8, [43, 44]), or at the '3rd International Conference on Biomedical Electronics and Devices' (BIOSTEC, Valencia, Spain, 20-23 January, 2010; chapter 7, [46]). Chapter 6 represents Hadorn's proposal for a 'fellowship for prospective researchers' that passed the negotiation of the Swiss National Science Foundation in October 2009. The results of a productive collaboration between Boenzli, Hadorn and Eggenberger Hotz were reported at the 'International Conference on Engineering and Meta-Engineering' (ICEME, Orlando, Florida, USA, 6-9 April, 2010; Appendix 1, [47]). The approved manuscript is reprinted with the kind permission of Boenzli in the Appendix of this thesis. Chapters 3 to 8 and Appendix 1 are presented in publication style as they were published; only adjustments to the typefaces and reference style were made. Consequently, the chapters of this thesis will be considered as self-contained. To make the thesis readily accessible, the chapters are not chronological but they consecutively highlight programmability, information processing, and functionality.

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CHAPTER 2

'PROPER' MATERIAL PROPERTIES: COMPONENTS THAT OFFER PROGRAMMABILITY, INFORMATION PROCESSING, AND FUNCTIONALITY TO FPAACS

1 Preliminaries

Before starting, key decisions had to be made regarding which morphology and materials should embody artificial cells to allow for programmability of matter and morphology, information processing, and functionality.

1.1 Morphology

A cell is the basic unit of all known living creatures and represents the smallest unit that exhibits the properties of life. Every organism consists of several to myriad cells or is itself a single cell. In addition to 'cellularity', Walde [1] lists the following eight key features of all living cells: (i) a plasma membrane, which separates the interior of the cell from the environment, (ii) double-stranded DNA to store the genetic information, (iii) transcription and translation of the genetic information, (iv) metabolic processes that rely on enzymes, (v) metabolic processes that lead to cell growth, copying of genetic information, and cell division, (vi) ability to respond to stimuli, (vii) sizes between 1 and 30 μm, and (viii) a common type of chemistry.

Artificial vesicles, also called liposomes, represent the artificial system that comes closest to natural systems; they feature an aqueous compartment separated from an aqueous surrounding by a closed membrane built of natural phospholipids. Due to their structural composition in common with natural cells, vesicles are the most studied systems among biomimetic structures [2], providing a bottom-up procedure in the analysis of biological processes [3-5]. In addition, vesicles are applied in synthetics, where they are used both as mini-laboratories to study confined chemical reactions under biologically relevant conditions [6] and as bioreactors [7-9]. Their ability to control confinement, transport, and the manipulation of chemical cargo is used in vesicular drug delivery systems [10-12]. Hence, single vesicles are used in different types of analytic, synthetic, and medical applications.

Given the analogy to natural systems and compositional simplicity, researchers in the field of protocell research in general and those investigating LT in particular, focus on the creation of a single living cell and by extension, on one single individual compartment that integrates all cellular functions. Despite their prevalence, Eggenberger Hotz and Hadorn pursued another objective from the outset, that of investigating processes controlling the assembly of different compartments to form multi-vesicle systems of defined architecture instead of one single individual vessel whose task is to host all processes. Both the 'external compartmentalization' of multicellular organisms and the 'internal compartmentalization' of eukaryotic cells by organelles served as models for this morphology. Multicompartment systems offer a division of different membrane functions (confinement, biocompatibility, cargo release, targeting, and protection) among membranes of distinct compositions and dimensions. Specific chemical reactions can be segregated for the purposes of increased controllability, observability, stability, and biochemical efficiency by the restricted dissemination and efficient storage of reactants, and/or reaction products. Despite these advantages, tethered multi-vesicle systems have been applied only marginally in both bioreactor [13, 14] and cosmetic applications [15]. Due to their potential, they have been proposed several times as multicomponent or multifunctional drug delivery systems [16-19].

While the invention of multicellularity is considered one of the major transitions during the course of early evolution [20], its origin is intensively debated [21]. All multicellular life begins with a single fertilized egg cell. Based on the genetic information stored, this cell divides repeatedly to finally produce a multicellular organism of impressive complexity and precision. Because the genome is identical in every cell, cell differentiation is not caused by differences in the genetic information, but because cells express different sets of genes as a consequence of asymmetric cell division. Selective gene expression controls cell proliferation, cell specialization, cell interactions, and cell movement. Although all these processes are happening in parallel in a variety of different ways and in different parts of the organism, there is no central control; each cell in the developing embryo has to make its own decision based on its genetic instructions, its particular circumstances, and its memory, i.e., the components available in the cell lumen and the modifications of the genetic blueprint (cf., genomic imprinting).

Because approaches in LT are mainly restricted to single compartments and current protocols of replicating vesicles [22, 23] do not implement asymmetric cell division known to be important in morphogenesis [24, 25], self-organized self-assembly of distinct populations of vesicles, which are distinct in membrane composition and/or content, in assemblies of pre-defined and programmable architecture of vesicles have to take center stage to emulate cell differentiation during developmental processes of natural organisms resulting in spatially arranged and orchestrated cell types. Thus, experimental protocols should allow the specification of the vesicle content and provide vesicle assemblies of programmable architecture.

Concerning vesicle assemblies of programmable architecture, there are impressive examples of manually made vesicle networks [14, 26, 27]. However, for the implementation of man-made wetware that is versatile, robust, and adaptive and that allows for high-throughput analyses, self-assembly has to gain importance. Even though a wide variety of cross-linking interactions have been developed (see [28] for a recent review on molecular recognition of vesicles) and potential applications [29] have been formulated, multicompartment systems are introduced only marginally. This representation does not live up to the expectations that potential multicompartment systems are accredited with [17, 29, 30]. It is caused, however, by the absence of a selective and self-terminating adhesion mechanism able to link more than two distinct populations of vesicles.

1.2 Components

Phospholipids were chosen as the components to constitute the vesicle membranes. The vesicle were cross-linked into networks based on the hybridization of two complementary single stranded deoxyribonucleic acids (ssDNA) that were modified with biotin and anchored to the vesicular surface via streptavidin and phospholipid-grafted biotinylated polyethylene glycol (PEG) tethers.

1.2.1 Vesicle Membrane

Both biological and artificial vesicles feature an aqueous compartment partitioned from an aqueous surrounding by a lipid membrane that is nearly impermeable to hydrophilic substances. The lipid membrane consists of amphiphilic phospholipids that link a hydrophilic head and a lipophilic tail. Suspended phospholipids self-assemble to form closed, self-sealing solvent-filled vesicles that are bound by a two-layered sheet (a bilayer) of 6 nm in width, with all of their tails pointing toward the center of the bilayer. This molecular arrangement excludes water from the center of the sheet and thereby eliminates unfavorable contacts between water and the lipophilic (= hydrophobic) tails of the phospholipids. The lipid bilayer provides inherent self-repair characteristics due to lateral mobility of its phospholipids [31] (for a recent review in the organization of lipid membranes see [32]).

Biological membranes are a fluid mosaic of lipids and other molecules; the richness of their chemical and mechanical properties *in vivo* is often dictated by an asymmetric distribution of these molecules. Current *in vitro* vesicles procedures in the laboratory based on a variety of methods including sonication [33], extrusion [34], swelling [35], electroformation [36], and reverse evaporation [37] preclude such asymmetric structures, because they produce symmetric vesicles in leaflets as well as in *intra*- and *inter*vesicular media (for a recent review in vesicle formation techniques see [14]). Vesicle formation protocols that allow asymmetry in leaflet composition [38] and media [8, 39-41] were reported. The independent formation of phospholipid monolayers resulting in primary asymmetric leaflets potentially made of completely different types of molecules could increase the flexibility, biocompatibility, and endurance of vesicles as drug delivery systems, as well as the applicability of vesicles in engineering novel types of composite bilayers.

The incorporation of phospholipid-grafted PEG to the vesicles membrane both stabilizes vesicles in blood circulation and induces increased membrane permeability [42]. Concerning vesicle aggregation, the use of long and flexible biotinylated phospholipid-grafted PEG tethers (bPEG-phospholipids) offers a higher detachment resistance than short and rigid spacers [43], as well as an absence of intermembrane transfer of linkers [44, 45].

1.2.2 Vesicle Linkage

Virtually all vertebrate cells express one or more cell adhesion molecules (CAMs), which enable cells to selectively adhere. Adhesion is selective to such an extent that embryonic tissues that are artificially mingled spontaneously sort out and restore their normal arrangement [46]. CAMs provide a multitude of distinct linkers and hence selectivity to the adhesion process. In addition to adhesive affinities and association constants of CAMs, Steinberg [47] reported that the mere abundance of CAMs contribute to the specification both of the cells' morphogenetic behavior and of the anatomical structures which the cells tend to organize into when assembled. During cell differentiation, the specificity of cell adhesion gradually increases. Moreover, recent data suggest that adhesion is tightly and dynamically regulated to allow directed cell locomotion [48]. Like a Velcro fastener that is composed of distinct populations of complementary hooks and loops, the binding characteristics of each cell or cell type is defined at any given time.

Vesicle linkage can be traced back to the pioneering work of Chiruvolu *et al.* [30] who used site-specific ligand-receptor (biotin-streptavidin) coupling to induce higher order self-assembly of vesicles. Since then, the multivalent Velcro-like interactions induced by CAMs are emulated in artificial systems by a multitude of different linking mechanisms [30, 49-57] (for the latest developments in biomimetic supramolecular chemistry see [28]). The multivalent, selective and sequence-dependent linkage of nucleic acids is credited with considerable potential because it mimics CAMs the best and offers programmability to the self-assembly process.

1.2.2.1 Streptavidin

To link biotin-ssDNA to the biotinylated vesicle membrane (bPEG-phospholipids), streptavidin was chosen as the connector. Streptavidin is a tetramer and thus provides four biotin binding sites [58]. The biotin-streptavidin system offers the strongest noncovalent biological interaction known [59]. Moreover, component modularity is provided by the universality of the biotin modification of myriads of substances. Due to the fluid character of the membrane, linkers accumulate at the adhesion sites [18, 49, 56, 60-64], thus increasing adhesion strength and offering self-termination to the self-assembly process. The adhesion strength is increased and the intermembrane transfer of linkers is also reduced by the double anchorage and double linkage per streptavidin tetramer, as well as the linkage-independent streptavidin crystallization [65].

1.2.2.2 Single Stranded DNA

Nucleic acids are not naturally found exposed on the membranes of cells. However, in artificial systems, single strands of DNA are used either to induce assembly of hard sphere colloids [66-69] or vesicles [45, 70], to induce the programmable fusion of vesicles [71, 72], or to spontaneously and specifically link vesicles to surface-supported membranes [45, 71, 73-77]. However, a linkage of more than two populations of vesicles was not implemented. Eggenberger Hotz and Hadorn remedied this lack only recently by the implementation of a DNA-mediated self-assembly of three distinct populations of vesicles [78].

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CHAPTER 3

PROGRAMMABILITY OF MATTER: TO ALLOW FOR SPECIFICITY OF THE SELF-ASSEMBLY PROCESS

Publication Profile	
Title:	Effect of Monovalent Inorganic Ions on Artificial Vesicles
Authors:	Hadorn M, Eggenberger Hotz P
Maik Hadorn Contributions:	Conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared the figures and tables.

Effect of Monovalent Inorganic Ions on Artificial Vesicles

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ABSTRACT. Due to the sequence-specific nature of DNA pairing, DNA represents an ideal candidate to act as a linker in multi-vesicle assemblies of programmable composition and spatial arrangements. Ionic species present during DNA-mediated self-assembly are boon and bane – they allow for DNA pairing while destabilizing vesicles. In this study, we, therefore, used a recently reported high-throughput method to screen for inorganic ions that cope with this trade-off situation. Sodium iodide was identified as the monovalent inorganic species reducing vesicle stability the least. In other words, we provide a sound basis for the specific DNA-mediated self-assembly of multi-vesicle structures.

KEYWORDS. DNA-mediated self-assembly, multi-vesicle structures, monovalent inorganic ions, destabilization, sodium iodide

INTRODUCTION

The formation of multi-vesicle complexes was introduced by Chiruvolu *et al.* about 15 years ago [1]. The programmable self-assembly of multi-vesicle complexes composed of several distinct entities has attracted significant attention in nanotechnological applications [2-10]. The pairing of DNA single strands depends on the base composition and the sequence of bases along the single polymer chains. It, therefore, represents an ideal candidate for the implementation of multi-vesicle assemblies of programmable composition and spatial arrangement. Thus, DNA was introduced as a cross-linking agent to induce the assembly of complementary monohomophilic hard sphere colloids [11-14] or vesicles [15-17], to induce programmable fusion of vesicles [16, 18], or to the spontaneously and specifically link vesicles to surface-supported membranes [15, 16, 19-22].

The formation and stability of DNA double strands (dsDNA) not only depends on the inherent base sequence but, it is also influenced by external parameters such as temperature, solvent, pH, and ionic strength [23]. However, mono- and divalent ions destabilize vesicles resulting in an increase in rates of vesicle fusion and rupture. This phenomenon is exploited in the formation of supported lipid bilayers (SLB) [24], which have received increasing practical and scientific interest due to their application potential in the life sciences [25]. To provide specificity to the DNA-mediated self-assembly process, one faces the following trade-off problem: ionic species have to be present during self-assembly to allow for DNA pairing, while negative effects of ions on vesicle stability inducing unspecific adhesion and fusion or hemifusion [26] have to be minimized to provide specificity.

Surface chemistry and surface charge, lipid headgroup charge, temperature, osmotic pressure, and the type and concentration of ions influence the formation of SLBs [27-34]. In particular, the effect of mono- and divalent cations on biological and artificial membranes is well studied *in silico* [35-46], indicating that cations actually interact with the lipids quite strongly. However, a systematic study on the influence of mono- and divalent cations, as well as of anions was reported recently by Seantier and Kasemo [47] and Leontidis *et al.* [48].

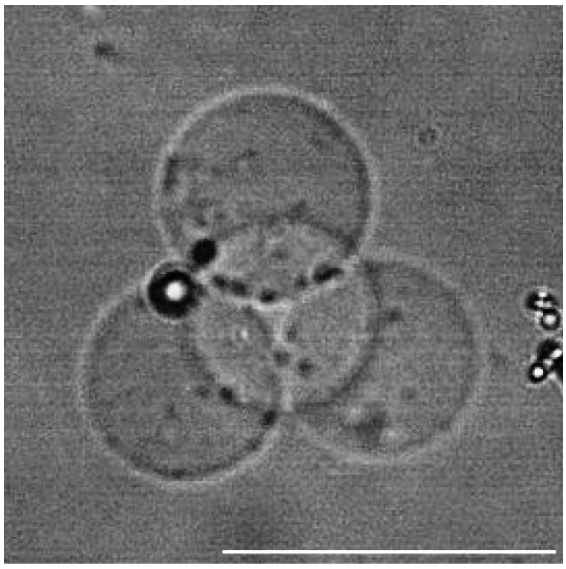

Fig. 1. *Sodium chloride induces the unspecific linkage of manually assembled vesicle tetrahedron.* The four vesicles originated from one population and were chosen to correspond in size. Their surface was not covered by any specific linkers (e.g., DNA single strands). Unspecific vesicle linkage was induced by increasing the osmotic pressure (addition of highly concentrated sodium chloride) of the hosting medium after the vesicle had been positioned by micromanipulation. Stable but unspecific vesicle linkage may be induced by sodium chloride. Light-micrograph; scale bar represents 100µm.

We already exploited the unspecific fusion or hemifusion induced by osmotic stress in the assembly of a vesicle tetrahedron (Figure 1). Concerning the DNA-mediated self-assembly process, the specificity was lost when using sodium chloride (Figure 2). Previously, we established a high-throughput method to test for effects of mono- and divalent organic sodium ions on the formation and stability of artificial vesicles [49]. The adsorption of vesicles to polystyrene surfaces indicates a higher probability of unspecific vesicle fusion or hemifusion. In this study, the same methodology was used to identify the monovalent inorganic species that least hindered vesicle stability. Examples of both kosmotropic (hydrophilic) and chaotropic (hydrophobic) cations and anions such as rubidium, potassium, sodium and lithium salts of chloride which follow the Hofmeister series, as well as fluoride and iodide sodium salts, were tested. Chloride is considered the dividing anion between the kosmotropes and the chaotropes [48, 50, 51]; fluoride is predicted to be completely excluded from the lipid bilayer [48], whereas iodide is chaotropic.

MATERIALS AND METHODS

For a schematic illustration of the vesicle formation technique and technical terms used see [52]. For modifications see [53-55]. Briefly, the modification involved the introduction of microplates (U96 MicroWell™ plates, polystyrene clear, U-bottom, Thermo Fisher Scientific, Langenselbold, Germany) to increase procedural manageability in laboratory experimentation and introduction of a density difference between the *inter-* and *intra*vesicular solutions, to detach the vesicles from the interface between 'intermediate' and 'aqueous phase'. The

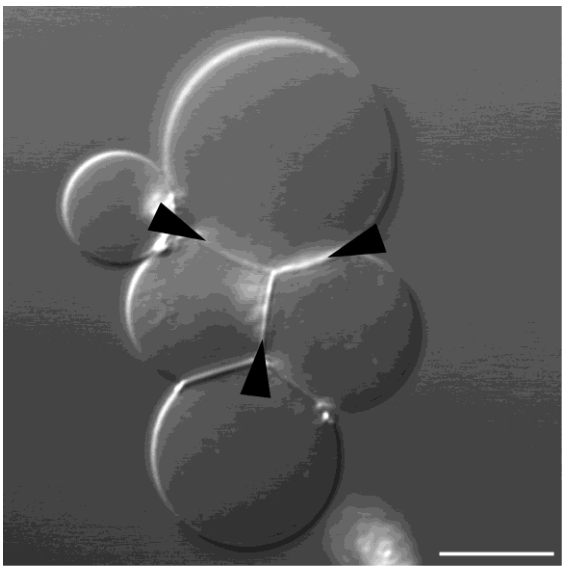

Fig. 2. *Sodium chloride counters the specificity of the DNA-mediated self-assembly process.* Two vesicle populations covered by DNA single strands of complementary sequence were merged. The flattened interfaces indicate vesicle linkage. Because vesicle coverage is restricted to either the sense or the antisense strand, the occurrence of triplets (or structures of higher order) with three bilateral adhesion sites (arrows) was unexpected. Because the exchange of DNA linkers between the vesicle populations was never observed, unspecific vesicle linkage was induced by sodium chloride (the intra- and intervesicular fluid contained 137 mM sodium chloride). Differential interference contrast micrograph; scale bar represents 10 µm.

Table 1. Relative liposome yield in relation to inorganic sodium salts. The liposome yield is expressed as a percentage of the control (*intra*-liposomal fluid without the addition of salt).

	50 millimolar	10 millimolar
Positive control H_2O	100.000 ± 6.205	100.000 ± 6.530
LiCl Lithium chloride	37.708 ± 52.206	0
NaCl Sodium chloride	0	45.775 ± 63.306
KCl Potassium chloride	0	95.930 ± 6.231
RbCl Rubidium chloride	0	99.340 ± 6.231
NaF Sodium fluoride	0	95.246 ± 5.491
NaI Sodium iodide	88.023 ± 2.734	96.240 ± 6.530

'emulsion phase' was prepared from sucrose (control, solution S1; sucrose BioUltra, Sigma-Aldrich, Buchs, Switzerland) or sucrose and lithium chloride (S2, LiCl), potassium chloride (S4, KCl), rubidium chloride (S5, RbCl), sodium fluoride (S6, NaF), sodium iodide (S7, NaI; all salts were purchased from Sigma-Aldrich, Buchs, Switzerland and used without further purification) or sodium chloride (S3, NaCl; Carl Roth GmbH, Karlsruhe, Germany). The phospholipid 2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (POPC, Sigma-Aldrich, Buchs, Switzerland) was purchased as a powder, dissolved in chloroform (Scharlau, Barcelona, Spain) to a concentration of 5 mg/ml upon arrival and, after chloroform evaporation (under vacuum, 60 min), was dissolved in mineral oil (light, Sigma-Aldrich, Buchs, Switzerland) to a final concentration of 200 µM and used to produce the 'intermediate phase' and the 'emulsion phase'. The POPC dissolved in chloroform was kept at -20 °C until further use. The POPC dissolved in mineral oil was used within a few days. The 'aqueous phase' was prepared from 1000 mOsm glucose and the same inorganic ions as the 'emulsion phase'. The water-in-oil emulsion of the 'emulsion phase' was equiosmolar to the 'aqueous phase' and was prepared in microtubes by adding 20 µl of solutions S1 to S7 to 1 ml of POPC dissolved in mineral oil. The mixture was mechanically agitated, sonicated three times for five seconds and placed over the 'intermediate phase' (100 µl placed over 100 µl of 'aqueous phase'). After incubation (10 min, room temperature), centrifugation (1500 × g, 15 min, 4 °C) induced vesicle formation. Due to the density difference of the *inter-* and *intra*-liposomal fluid and the geometry of the microplate bottom, the vesicles pelletized in the center of the well. Vesicle formation was performed in duplicates. The length of circumference of the vesicle pellet was used as a measure of vesicle yield (cf., methodology in [49, 55]), but data of length of circumference were only collected if vesicles pelletized. The vesicle yield was compared to the control by providing values of relative vesicle yield. Light microscopy was performed using a Wild M40 inverted microscope equipped with a MikoOkular microscope camera. All camera settings were identical for the recordings.

RESULTS AND DISCUSSION

The vesicles were found to sediment and were, therefore, easily available for inverse microscopy. The inorganic ions tested (Table 1) revealed noteworthy and non-trivial effects. The relative vesicle

Fig. 3. (opposite) *Of all monovalent ions tested, sodium iodide reduces vesicle stability the least.* Monovalent ions were present during vesicle preparation in the intra- und intervesicular fluid. The density difference between the intra- and intervesicular fluid, centrifugation, and the geometry of the hosting micro well induced vesicles to accumulate at the bottom and the center of the hosting well (cf., control). The length of the circumference of the vesicle pellet was used as a measure of vesicle yield. If the ionic species prevented either vesicle formation or pelletization (visually inspected), the length of circumference was set to 0. Size-width: 2.5 mm; size-height: 2 mm; image details are of 3× digital magnification.

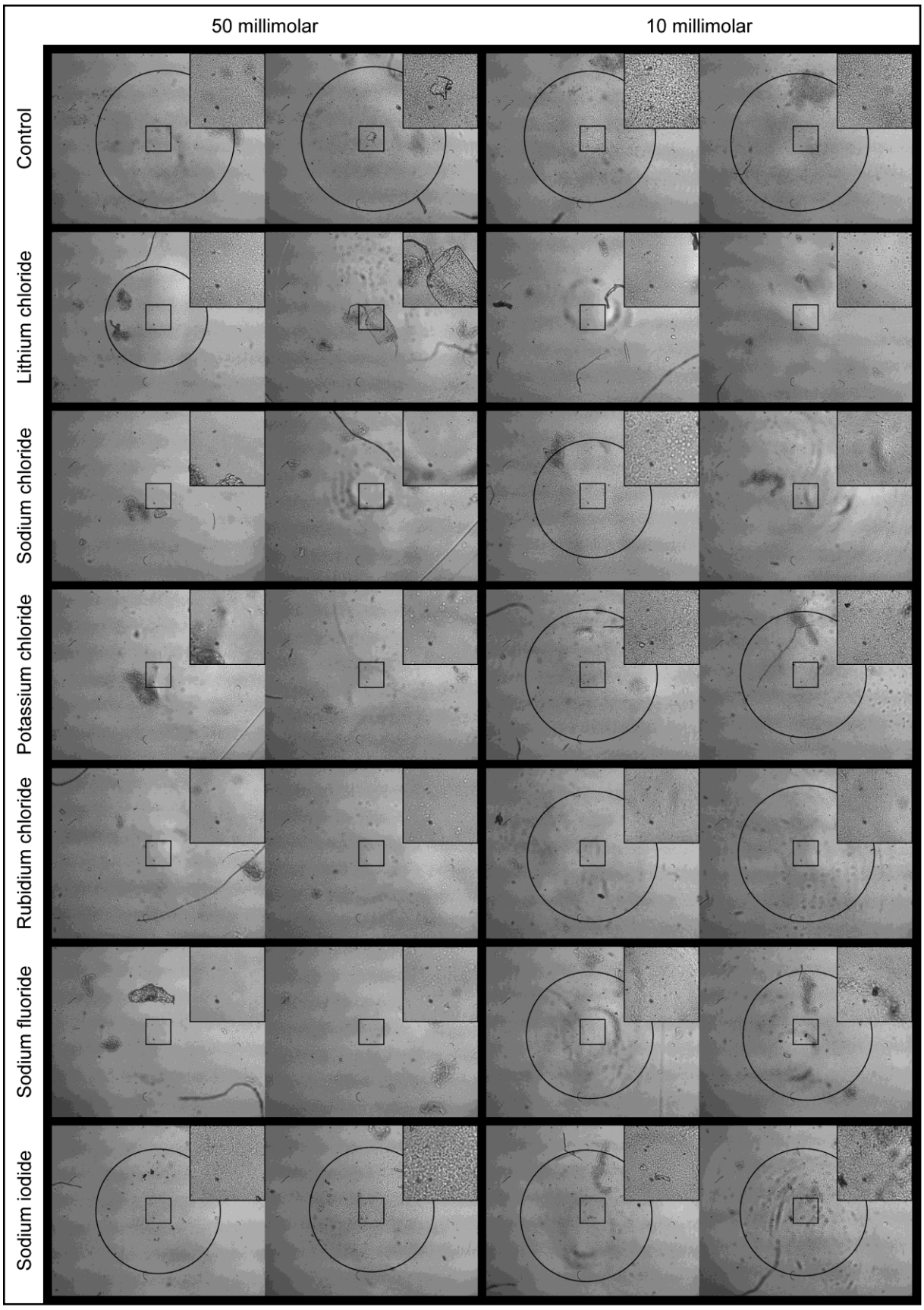

Fig. 3. *Sodium iodide reduces vesicle stability the least of all monovalent ions tested.*

yield was significantly different, but it depended on the concentration of the sodium salt (Figure 3). At a concentration of 10 mM, the vesicle yield was almost as high as in the control experiment; the exceptions were lithium and sodium chloride. At 50 mM, vesicle production and/or vesicle pelletization only tolerated sodium iodide. Hence, sodium iodide is the monovalent inorganic species reducing vesicle stability and/or pelletization the least of the salts tested.

The sample size was restricted to provide high-throughput and allow a large number of ions to be tested in parallel on the same plate. However the high variance in vesicle stability and/or pelletization is representative for sodium chloride; it may also be representative for lithium chloride, because the variability in vesicle handling was persistent for numerous self-assembly experiments that used sodium (chloride) to compensate for the high negative charge of the sugar-phosphate DNA backbone, thus allowing DNA pairing. Vesicle stability and/or pelletization are negatively correlated to the Hofmeister series for cations, but correlate positively when it comes to anions. This non-trivial finding was unexpected and awaits further investigations.

Another open question is the lack of ion-related drawbacks when dealing with vesicle formation by the centrifugation method [52, 56], or DNA-mediated self-assembly of vesicles prepared by electroformation [15, 17]. Acetate [56] was found to affect vesicle formation and/or pelletization to a lesser extent [49]; sodium (chloride), however, is known to induce unspecific vesicle adhesion, fusion, and rupture (cf., [28]). Vesicles undergo only modest mechanical stresses in [52], due to the lack of density difference between the *intra*- and *inter*vesicular fluid, when compared to the modified vesicle formation procedure used here. Vesicles prepared by the centrifugation method in [52, 56] may, therefore, be more stable and/or do not face any solid surfaces. However, the influence of sodium on the specificity of the DNA-mediated vesicle self-assembly process needs a closer look. In particular, Beales and Vanderlick reported a lack of specificity and difficulties in unbinding giant vesicles by thermal dehybridization for the DNA-mediated self-assembly process (cf., discussion in [15]). Moreover, when comparing examples of vesicle clusters (see Figure 3 in [15]) equal in DNA coverage (5.0×10^{-3} ssDNA/lipid) but distinct in sodium chloride concentration, it is unclear why 38 mM sodium chloride induces small vesicle clusters, whereas 55 mM results in large assemblies, because the predicted melting temperatures (T_m); using the %GC method [57, 58] differ by 2.6 to 3 °C (predicted T_m: 38mM Na^+: 25, 28 °C; 55mM Na^+: 27.6, 31). We interpret these findings as follows: The DNA-mediated self-assembly process depends exclusively on the sodium chloride concentration that induces unspecific vesicle assemblies. This interpretation becomes even more feasible when comparing small assemblies resulting from a surface coverage of 5.0×10^{-3} ssDNA/lipid (38 mM NaCl, predicted T_m: 25, 28 °C) with large assemblies of vesicles covered by 1.3×10^{-3} ssDNA/lipid (47 mM NaCl, predicted T_m: 26.5, 30 °C).

CONCLUSIONS

Of the tested ions, sodium iodide is the most promising monovalent ionic species, because it reduces vesicle stability the least. To find the optimal balance between enabling DNA pairing and reducing the destabilization of vesicles, the effect of different sodium iodide concentrations on the DNA-mediated self-assembly of artificial vesicles was studied systematically in the following experiments [59].

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CHAPTER 4

PROGRAMMABILITY OF MATTER: TO ANALYZE SPECIFICITY OF THE SELF-ASSEMBLY PROCESS

Publication Profile	
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Pages:	e9886
URL:	http://dx.doi.org/10.1371%2Fjournal.pone.0009886
Maik Hadorn Contributions:	Conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared the figures and tables.

Reviewer Comments

---------- review 1 ----------

For instance the effect of ligand (oligonucleotide) loading and the stability of the aggregates should be addressed.

The authors also argue that simple anchoring of the oligonucleotides via lipids does not provide sufficient stability. However, the present system is also anchored to the vesicles via lipids. Thus is not obvious what the great advantage is.

---------- review 2 ----------

It would be nice to see experimental evidence for the programmability of the authors' method for making multi-vesicle systems. This is clearly an expected benefit of using ssDNA, but I did not notice any evidence of it here. It would be great to provide evidence that suggests that you could program the assemblies in an open-ended combinatorial way. E.g., the first step would be to compare a simple and a more complex vesicle assembly, demonstrating a gradually increasing aggregate complexity. Short of that, it would be good to explain exactly to what extent this is achieved or can be confidently anticipated on the basis of the results demonstrated here.

The last figure (8 micrographs) is a crucial part of the results reported here, but I have two problems with this figure: First, the layout is supposed to correspond to some part of Figure 1. But I am confused about how Figure 1 corresponds to the last figure. Making this clear would be a big improvement in the paper.

The last figure (8 micrographs) is a crucial part of the results reported here, but I have two problems with this figure: Second, the main point of the last figure (e.g., lines 479-493) is to demonstrate the authors' method of forming programmable multivesicle structures, and the figure caption refers to these structures (plagues etc.). I see the plaques in C.1 (arrows) But I have a hard time clearly seeing these multivesicle structures in any of the panels.

E.g., the vesicles in B.1 seem more "squashed together" than in B.2, but what exactly does this show, if anything?

One final point. Some of the motivations for the authors' work concern hypothetical future accomplishments. The authors should be more clear about what has been achieved and what is only hypothetical today. E.g., the medical application mentioned is a hypothesis about the future. But is programming consecutive chemical reactions achieved today, or is it just a hypothesis. This should be clarified.

Can you see any *evidence* in your micrographs of the general programmability of the multicomponent assemblies? Or evidence of a concrete step in this direction? This is not clear to me.

---------- review 3 ----------

The authors should provide some (even speculative) explanation concerning the absence of DNA-independent vesicles aggregation mediated by streptavidin alone (i.e. biotinylated vesicle - streptavidin - biotinylated vesicle).

Finally, picture colors and icons of Figure 1 should be chosen carefully to facilitate readability. For instance DNA icon and Strepavidin-AF488 and streptavidin color are hardly distinguishable even when printed in high resolution.

DNA-Mediated Self-Assembly of Artificial Vesicles

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Background. Although multicompartment systems made of single unilamellar vesicles offer the potential to outperform single compartment systems widely used in analytic, synthetic, and medical applications, their use has remained marginal to date. On the one hand, this can be attributed to the binary character of the majority of the current tethering protocols that impedes the implementation of real *multi*component or *multi*functional systems. On the other hand, the few tethering protocols theoretically providing *multi*compartment systems composed of several distinct vesicle populations suffer from the readjustment of the vesicle formation procedure as well as from the loss of specificity of the linking mechanism over time.

Methodology/Principal Findings. In previous studies, we presented implementations of *multi*compartment systems and resolved the readjustment of the vesicle formation procedure as well as the loss of specificity by using linkers consisting of biotinylated DNA single strands that were anchored to phospholipid-grafted biotinylated PEG tethers via streptavidin as a connector. The systematic analysis presented herein provides evidences for the incorporation of phospholipid-grafted biotinylated PEG tethers to the vesicle membrane during vesicle formation, providing specific anchoring sites for the streptavidin loading of the vesicle membrane. Furthermore, DNA-mediated vesicle-vesicle self-assembly was found to be sequence-dependent and to depend on the presence of monovalent salts.

Conclusions/Significance. This study provides a solid basis for the implementation of multi-vesicle assemblies that may affect at least three distinct domains. (i) Analysis. Starting with a minimal system, the complexity of a bottom-up system is increased gradually facilitating the understanding of the components and their interaction. (ii) Synthesis. Consecutive reactions may be implemented in networks of vesicles that outperform current single compartment bioreactors in versatility and productivity. (iii) Personalized medicine. Transport and targeting of long-lived, pharmacologically inert prodrugs and their conversion to short-lived, active drug molecules directly at the site of action may be accomplished if multi-vesicle assemblies of predefined architecture are used.

INTRODUCTION

Artificial vesicles feature an aqueous compartment separated from an aqueous surrounding by a closed membrane that is almost impermeable for hydrophilic substances. Like cell membranes, vesicle membranes consist of amphiphilic phospholipids that link a hydrophilic head and a lipophilic tail. All tails pointing towards the center of the membrane resulting in a two-layered sheet (a bilayer). This molecular arrangement excludes water from the center of the sheet thereby eliminating entropic unfavorable contacts between water and the lipophilic (= hydrophobic) tails. The lipid bilayer organizes processes by compartmentalizing them and provides inherent self-repair characteristics due to lateral mobility of its phospholipids [1].

As a result of the analogy to natural systems and the compositional simplicity, artificial vesicles are the most studied systems among biomimetic structures [2] providing a bottom-up procedure in the analysis of biological processes [3-5]. In addition,

vesicles are applied in synthetics where they are used both as mini-laboratories to study confined chemical reactions under biologically relevant conditions [6] and as bioreactors [7-9]. Their ability to control confinement, transport, and manipulation of chemical cargo is used in vesicular drug delivery systems [10-12]. Single unilamellar vesicles are used essentially in analytic, synthetic, and medical applications. In contrast, multicompartment systems offer a division of different membrane functions (confinement, biocompatibility, cargo release, targeting, protection) among membranes of distinct compositions and dimensions. Specific chemical reactions can be segregated for the purposes of increased controllability, observability, stability, and biochemical efficiency by restricted dissemination and efficient storage of reactants, and/or reaction products. Thus, tethered multi-vesicle systems have been realized in both bioreactor [13, 14] and cosmetic applications [15] and proposed as multicomponent or multifunctional drug delivery systems [16-19]. The authors already discussed potential applications of multi-vesicle systems in personalized drug delivery [20] and as real-world testbeds of results observed *in silico* [21].

Current tethering protocols of multi-vesicle systems are based either on electrostatic or donor-acceptor interactions [22-31]. Due to the binary character of these linking mechanisms real *multi*component or *multi*functional systems are hardly feasible. The programmable self-assembly of superstructures composed of *n* distinct entities with high degrees of complexity [32] has attracted significant attention in nanotechnological applications [33-36]. Since single stranded DNA (ssDNA) offers a multitude of distinct linkers, high specificity of binding between complementary sequences, and a digital nature of DNA base coding, it represents an ideal candidate for the implementation of multi-vesicle assemblies of programmable composition and spatial arrangement. DNA single strands were therefore introduced as crosslinking agents to induce the assembly of complementary monohomophilic hard sphere [34, 37-39] or vesicle [40, 41] colloids, to induce programmable fusion of vesicles [41, 42], or to spontaneously and specifically link vesicles to surface supported membranes [40, 41, 43-46]. However, current DNA-mediated linking mechanisms suffer from two shortcomings. In most cases, linkers are composed of ssDNA covalently linked to cholesterol [40, 43, 45, 46] or to lipids [41, 44]. Single cholesterol-tagged ssDNA (monocholesterol ssDNA) spontane-

Fig. 1. (opposite) *Schematic representation of experimental setups A, B, and C.* Numbers (0-3) indicate processes and small letters (a-e) indicate states. (Setup A) The incorporation of phospholipid-grafted PEG tethers into the vesicle membrane is analyzed. Vesicle populations (VPs) differ in the presence (VP A1) and absence (VP A2) of phospholipid-grafted fluorescently labeled PEG tethers (cfPEG2000-DSPE) during vesicle formation. (Setup B) To settle the specificity of membrane loading with streptavidin depending on the presence of anchoring sites, phospholipid-grafted biotinylated PEG tethers (bPEG2000-DSPE) are either present (VP B1) or absent (VP B2) during vesicle formation. Both VPs are subsequently incubated with fluorescently labeled streptavidin. Excess streptavidin is removed after incubation. (Setup C) To designate both the sequence-dependence and the dependence on the monovalent salt concentration of the vesicle self-assembly process two VPs either loaded with complementary (VP C1, VP C2) or noncomplementary (VP C3, VP C4) DNA single strands (ssDNA) are unified in solutions distinct in sodium iodide concentration. The streptavidin solutions were individually preincubated with biotin-ssDNA solutions prior to vesicle decoration (see microtubes holding the streptavidin/biotin-ssDNA solutions). After incubation of vesicles excess streptavidin/biotin-ssDNA is removed. DNA hybridization of complementary ssDNA causes accumulation of linkers and of the fluorescence signal (e.i) in the contact area over time (d to e) that is absent for noncomplementary ssDNAs (e.ii). (e.i) DNA-independent crystallization of streptavidin molecules on the surface of vesicles (*) that distributes stresses arising during/after DNA-mediated self-assembly may stabilize the linking system by compensating streptavidin molecules either incompletely equipped with biotin-ssDNA (**) or anchored only partially (***).

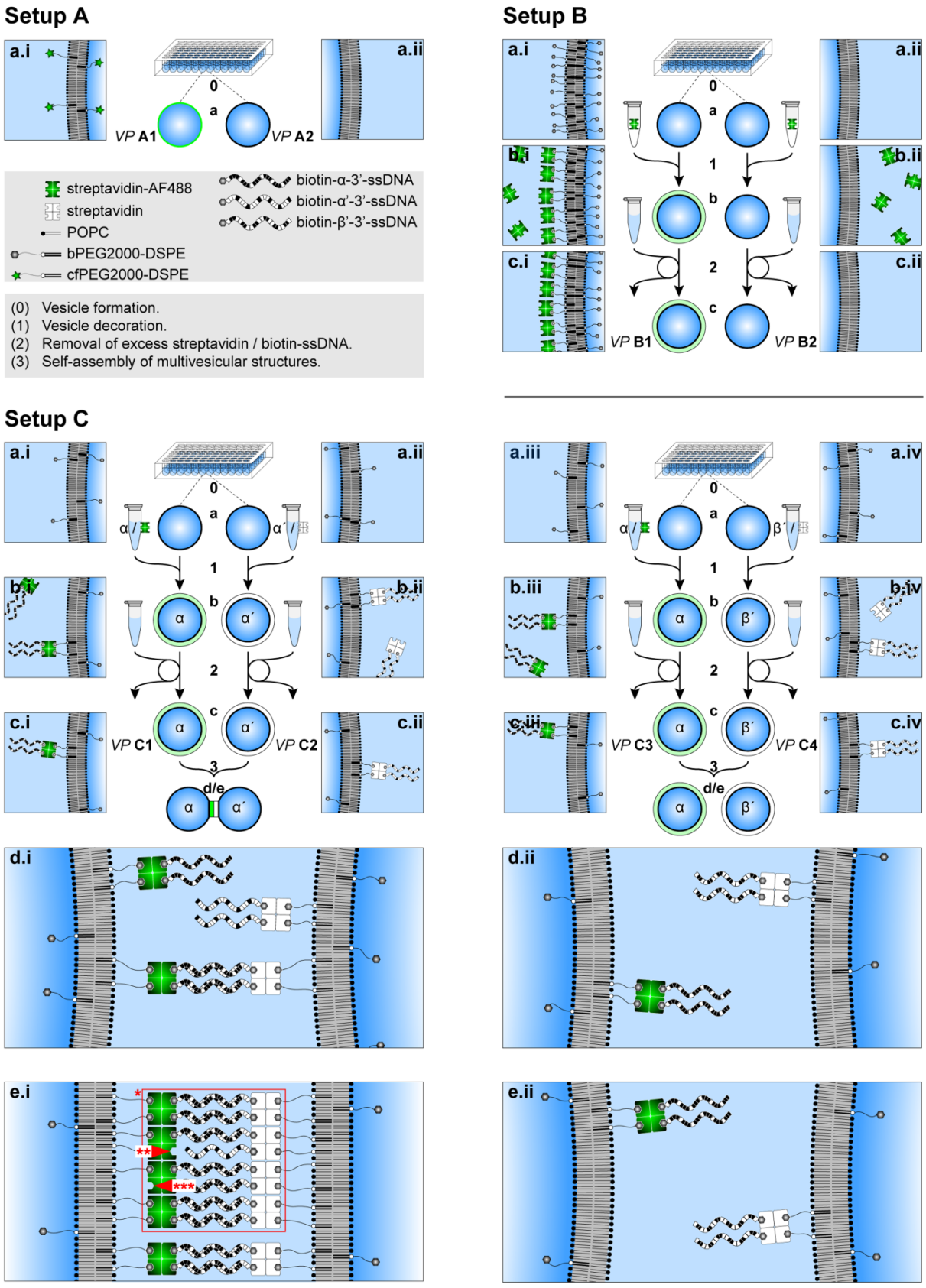

Fig. 1. *Schematic representation of experimental setups A, B, and C.*

ously leaves the lipid bilayer and incorporates randomly into (other) lipid bilayers [40, 47]. Thus, the specificity of the linking system is lost over time. Although this problem can be solved by using two anchors per ssDNA (e.g. bicholesterol ssDNA) [47] a second drawback remains intrinsic to the molecular architecture of the linkers. The partition coefficient of amphiphilic linkers is affected by the characteristics of their hydrophilic (ssDNA) and hydrophobic (membrane anchors) components. Thus, vesicle formation and/or composition have to be readjusted anew every time the characteristics (e.g. length of ssDNA) of the linkers are changed.

In previous work [20, 21, 48], we presented implementations of multicompartment systems and resolved the problem of readjusting vesicle formation/composition as well as of losing specificity by using linkers consisting of biotinylated DNA single strands (biotin-ssDNA) that were anchored by long and flexible phospholipid-grafted biotinylated PEG tethers via streptavidin as a connector. The problem linked to readjusting the vesicle formation procedure and/or vesicle composition was addressed by incorporating invariable and universal anchoring sites into the membrane during vesicle formation (phospholipid-grafted biotinylated PEG tethers). Since specificity is introduced only in a postprocessing step by strictly hydrophilic linkers (biotin-ssDNA linked to streptavidin) the vesicle formation procedure and vesicle composition can be kept uniform. Streptavidin is a tetrameric protein that provides two pairs of biotin-binding sites on opposite sides of each streptavidin molecule and that does not affect vesicle stability even if the surface of vesicles is completely coated with a monomolecular layer of streptavidin [49]. The biotin-streptavidin system offers the strongest non-covalent biological interaction known [50], a multitude of possible vesicle modifications, component modularity, and off-the-shelf availability. Since (i) the DNA strands are anchored by two phospholipid-grafted biotinylated PEG tethers per streptavidin molecule, (ii) the streptavidin crystallizes on the surface of vesicles [49, 51], and (iii) the phospholipid-grafted biotinylated PEG tethers provide high detachment resistance [52] and no detectable intermembrane transfer of linkers from donor liposomes to acceptor liposomes [53] it is reasonable to conclude that loss of specificity described for current DNA-mediated linking mechanisms remains absent for the tethering method presented in this study (see Figure 1, setup C, panel e.i for a schematic representation of factors that stabilize the linking system).

The lateral mobility of linkers results in a linkage-induced receptor accumulation at contact areas of adjacent and complementary vesicles [18, 22, 30, 54-58]. The depletion of linkers between the so-called adhesion plaques potentially terminates the (self-)assembly process and therefore defines the spatial arrangement of multi-vesicle aggregates [22, 40]. Thus, multi-vesicle aggregates may outperform hard sphere colloids not only in the ability of controlled confinement, transport, and manipulation of chemical cargo but also in the controllability of spatial organization by inherent material properties.

In the present study, we systematically analyzed (see Figure 1 for a detailed description) the single components of the DNA-based linking system by investigating the incorporation of phospholipid-grafted biotinylated PEG tethers to the vesicle membrane during vesicle formation (setup A), the streptavidin loading of the vesicle membrane in dependence of anchor sites concentration (setup B), and the specificity of the DNA-mediated vesicle-vesicle self-assembly in dependence of sequence complementarity and monovalent salt concentration (setup C). We subsequently discuss how multi-vesicle assemblies of predefined architecture may affect analysis, synthesis, and personalized medicine.

RESULTS AND DISCUSSION

Setup A: Incorporation of phospholipid-grafted biotinylated PEG tethers to the vesicle membrane. For microscopic analysis of the incorporation of phospholipid-grafted biotinylated PEG tethers to the vesicle membrane, biotin labeled PEG phospholipids (bPEG2000-DSPE) were replaced by carboxyfluorescein labeled PEG phospholipids (cfPEG2000-DSPE). A fluorescence signal was found exclusively at the vesicle membrane and only if fluorescently labeled phospholipids were present during vesicle formation (Figure 2). Thus, phospholipid-grafted biotinylated PEG tethers are incorporated into the

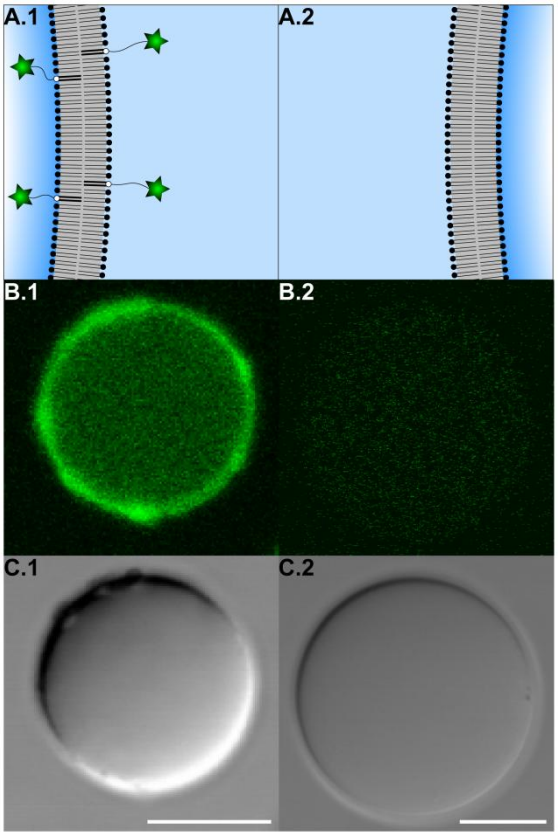

Fig. 2. *Experimental setup A: Incorporation of phospholipid-grafted biotinylated PEG tethers to the vesicle membrane.* (A) Schematic representation of the result of experimental setup A (cp. Figure 1, setup A, panels a.i and a.ii). (B) Confocal laser scanning fluorescence and (C) differential interference contrast micrographs of vesicle population (VP) A1 (left) and A2 (right). For a detailed description of the experimental setup see Figure 1 (setup A). VPs differed in the presence (VP A1) and absence (VP A2) of phospholipid-grafted fluorescently labeled PEG tethers (carboxyfluorescein, pseudocolored green in fluorescence micrographs) during vesicle formation. Scale bars represent 10μm.

vesicle membrane if present during vesicle formation.

Setup B: Streptavidin loading of the vesicle membrane in dependence of anchor sites concentration. Decoration of vesicle surfaces with fluorescently labeled streptavidin can be observed exclusively if anchoring sites – provided by bPEG2000-DSPE – were incorporated into the membrane during vesicle formation (Figure 3). Thus, specificity of decoration is provided by the presence of anchoring sites.

Setup C: Implementation of a DNA-mediated vesicle-vesicle self-assembly. Adhesion plaques (cp. Figure 4.D.1) emerging from DNA hybridization were found exclusively if DNA strands were complementary and sodium iodide was present at a concentration of 12.5 millimolar. At monovalent salt concentrations above 12.5 millimolar silhouette blurring (Figure 4.B.1, B.2) and a lower number of vesicles indicate reduced vesicle stability (lysis). Fur-

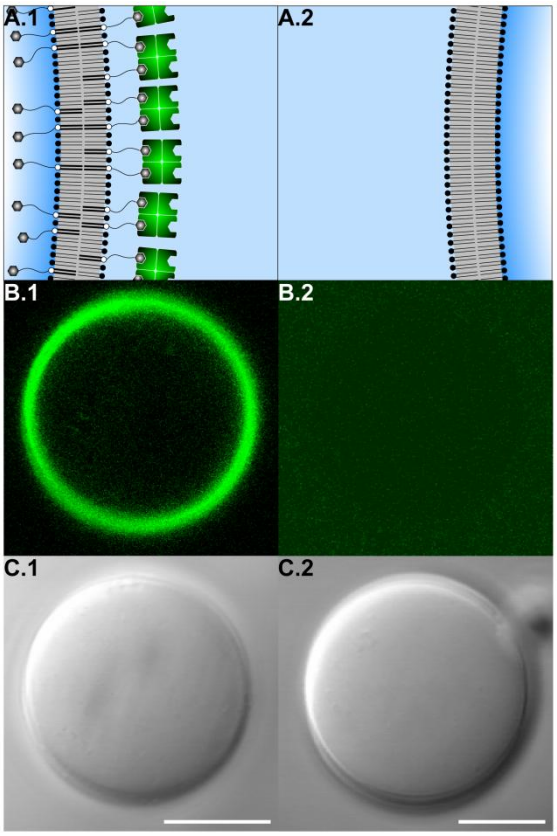

Fig. 3. *Experimental setup B: Streptavidin loading of the vesicle membrane in dependence of anchor sites concentration.* (A) Schematic representation of the result of experimental setup B (cp. Figure 1, setup B, panels c.i and c.ii). (B) Confocal laser scanning fluorescence and (C) differential interference contrast micrographs of vesicle population (VP) B1 (left) and B2 (right). For a detailed description of the experimental procedure see Figure 1 (setup B). VPs differed in the presence and absence of phospholipid-grafted biotinylated PEG tethers during vesicle formation. Both VPs were subsequently incubated with an excess of fluorescently labeled streptavidin (Alexa Fluor® 488, pseudocolored green in fluorescence micrographs). Scale bars represent 10μm.

thermore, DNA-independent vesicle-vesicle-linkage (Figure 4.C.2 (arrows)) as well as the emergence of a homogenous layer of interconnected vesicles (Figure 4.C.1) both indicate a loss of specificity of the adhesion process. Specificity therefore negatively correlates with monovalent salt concentration in the surrounding medium. In particular for two vesicles (*, ** in Figure 4.C.1) to be linked to a third one (***), vesicles * and ** have to present DNA strands of the same sequence on their surface theoretically inhibit their mutual linkage yet observed in Figure 4.C.1 (arrow). At a monovalent salt concentration of 12.5 millimolar, the formation of adhesion plaques depended on the complementarity of the DNA single strands (cp. Figure 4.D.1 vs. Figure 4.D.2) indicating specificity and hence programmability of the DNA-mediated self-assembly process. Since accumulation of fluorescently labeled streptavidin was absent if the DNA strands were not complementary (cp. Figure 4.D.2), both DNA-independent vesicle aggregation mediated by streptavidin [29] and linkage-independent crystallization of streptavidin [59] can be ignored as factor of adhesion plaque formation. Since a streptavidin molecule offers two pairs of biotin-binding sites, streptavidin and biotin-ssDNA concentrations were kept at a molar ratio of 1:2 during preincubation (prior to vesicle decoration, see Figure 4, setup C) to ensure that on average two binding sites were kept clear in order to link the streptavidin to the vesicle membranes. The absence of a DNA-independent vesicle aggregation mediated by streptavidin may be explained by the absence of free phospholipid-grafted biotinylated PEG tethers on the surface of the vesicle membranes after incubation with streptavidin. Fluorescence intensity was found to correlate positively with monovalent salt concentration in the surrounding medium. This dependence of binding efficiency of biotin-streptavidin on the concentration of sodium iodide is consistent with enhanced binding [60] and reduced dissociation efficiency [61] of streptavidin-biotin in the presence of mono- and divalent salts. An acceptable compromise between the binding efficiency of biotin-streptavidin and the specificity of the adhesion process was found in a salt concentration of 12.5 millimolar sodium iodide. In preliminary experiments (data not shown) vesicle aggregation was unspecific to a lesser extent and vesicles were more stable if sodium iodide was used instead of sodium chloride that is widely applied in vesicle self-assembly experiments. Due to the dependence of biotin-streptavidin binding on monovalent salt concentration, the well-known dependence of DNA hybridization on monovalent salt concentration [62, 63] could not be evaluated accurately herein. Since fluorescently labeled and unlabeled

Fig. 4. (opposite) *Experimental setup C: Implementation of a DNA-mediated vesicle-vesicle self-assembly.* (A) Schematic representation of the result of experimental setup C (cp. Figure 1, setup C, panels e.i and e.ii). (B-E) Confocal laser scanning fluorescence (left) and differential interference contrast (right) micrographs in two columns of merged vesicle populations (VPs) C1 to C4 (VP C1 & VP C2, VP C3 & VP C4). For a detailed description of the experimental procedure see Figure 1 (setup C). The biotinylated membranes (receptor surface density: 1.0 mol % biotin labeled PEG phospholipids) of all VPs were loaded with biotinylated single stranded DNA (biotin-ssDNA) using streptavidin as a cross-linking agent. VPs differed in streptavidin labeling (VPs C1/3: Alexa Fluor® 488, pseudocolored green in fluorescence micrographs, VPs C2/4: unlabeled) and biotin-ssDNA sequence (VP C1/3: α, VP C2: α', VP C4: β') – only sequences α and α' were complementary. Row headings indicate sodium iodide concentrations in the vesicle lumen and the surrounding medium. Fluorescence labeling of the membranes, silhouette blurring indicating vesicle lysis (cp. B.1, B.2), and accumulation of fluorescence signal positively correlate with sodium iodide concentration (microscope settings were identical for all pictures) causing a tradeoff between membrane loading, DNA hybridization, and vesicle stability. Adhesion plaques indicate stable vesicle-vesicle-linkage (visible adhesion plaques are highlighted in the differential interference contrast micrograph of D.1 by an image overlay with the confocal laser scanning fluorescence (processed) micrograph). The adhesion plaques of one vesicle (D.1) and DNA-independent vesicle-vesicle linkages (C.2) are highlighted by arrows. See text for a discussion of the loss of specificity of the DNA-mediated adhesion process observed in panels C.1 and C.2. Panel D.1 is reproduced with kind permission of Springer Science+Business Media (for original publication see [21]). Scale bars represent 10μm.

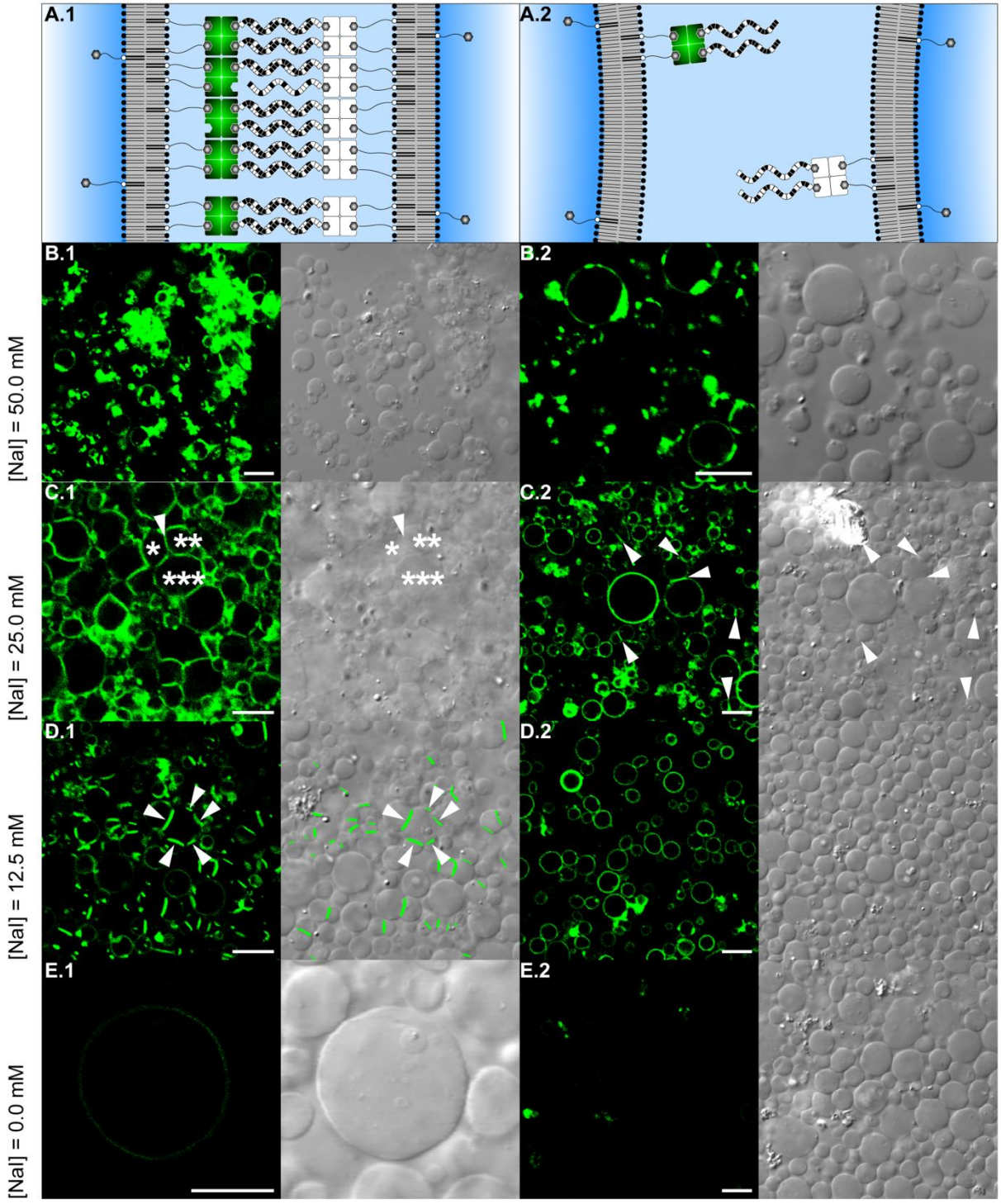

Fig. 4. *Experimental setup C: Implementation of a DNA-mediated vesicle-vesicle self-assembly.*

vesicles occurred approximately in equal numbers (cp. Figure 4.D.2) one can conclude that no transfer of linkers between the membranes of different vesicles occurred during experimentation (cp. [40, 47]). In the absence of monovalent ions, differences in fluorescence intensity between setups B and C (cp. Figure 4.B.1 vs. Figure 4.E.1) can be attributed to differences in the relative number of anchoring sites and distinct microscopic settings optimized to detect the weak fluorescence signal in Figure 4.B.1.

Theoretical predictions assume total streptavidin vesicle surface coverage and, as a consequence, absence of linker depletion between adhesion pla-

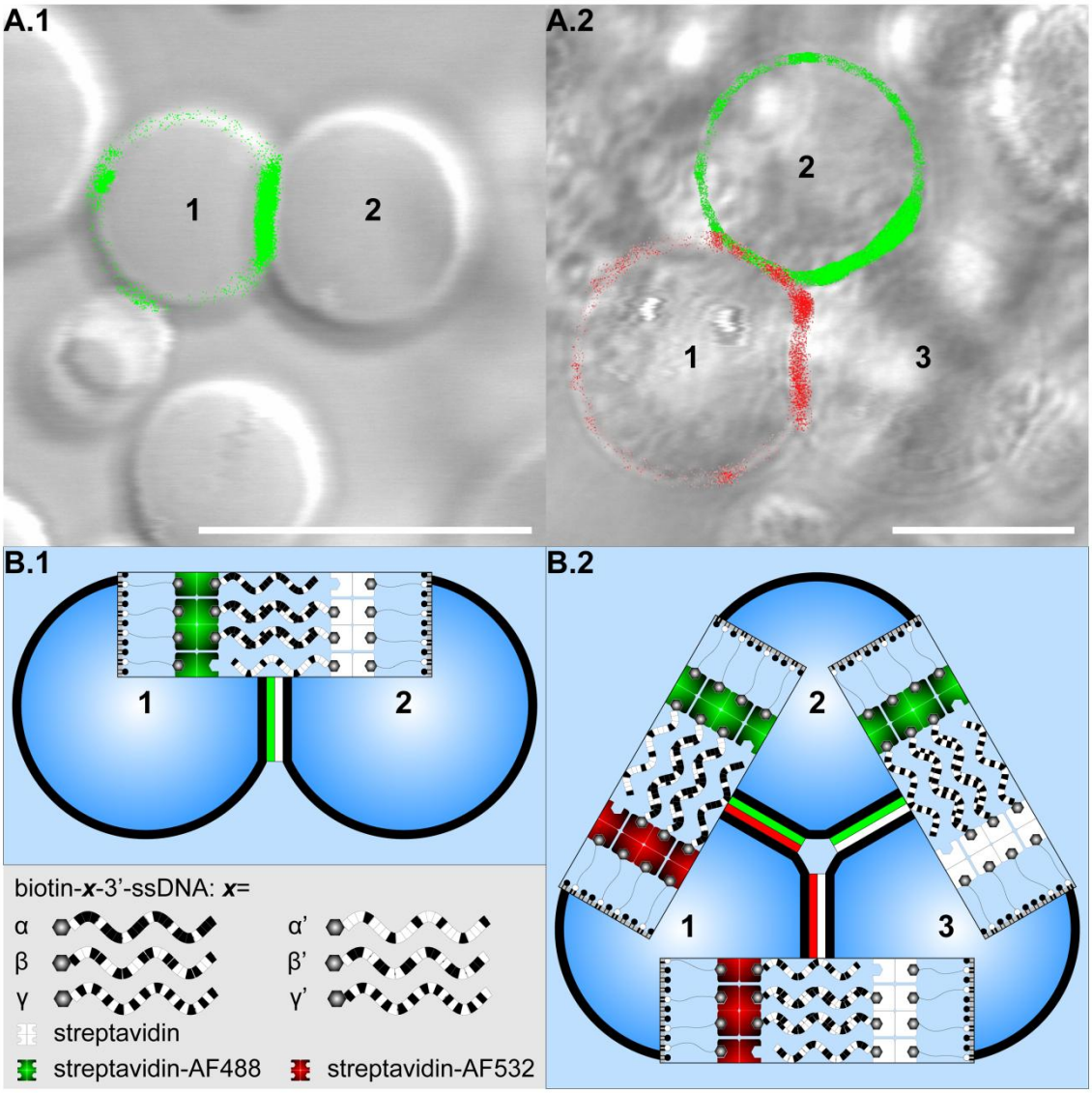

Fig. 5. *Programmability of the DNA-mediated self-assembly process: Multi-vesicle assemblies of predefined architecture.* (A) Image overlays of confocal laser scanning fluorescence and differential interference contrast micrographs of merged vesicle populations (VPs). Biotinylated DNA single strands (biotin-ssDNA) that differ in sequence (α, α′, β, β′, γ, γ′) and streptavidin populations that differ in fluorescence labeling (unlabeled (st.), Alexa Fluor® 488 labeled (st.-AF488, pseudocolored green in fluorescence micrographs), Alexa Fluor® 532 labeled (st.-AF532, pseudocolored red in fluorescence micrographs)) were incubated pairwise prior to vesicle decoration resulting in the following streptavidin/biotin-ssDNA combinations decorating the VPs: (A.1) α-st.-AF488: VP1, α′-st.:VP2; (A.2) α-st.-AF532 & β-st.-AF532: VP1, α′-st.-AF488 & γ-st.-AF488: VP2, β′-st. & γ′-st.: VP3. The receptor surface density was reduced to 0.25 mol % biotin labeled PEG phospholipids (cp. 1.0 mol % in experimental setup C). The fluorescence signal accumulates in the contact areas of adjacent and complementary vesicles forming adhesion plaques that indicate stable vesicle-vesicle linkage. (B) Schematic representation of the programmability of the DNA-mediated self-assembly process. The formation of adhesion plaques depends on the complementarity of ssDNA (cp. Figure 4.C.1) resulting in a sequence depend accumulation of linkers in the contact areas. The depletion of linkers in between the adhesion plaques terminates the self-assembly process. In combination with the ssDNA decoration of the vesicle surface, the self-termination defines the spatial arrangement of multi-vesicle aggregates. Thus, control of the assembly process is inherent to the system resulting either in duplets (A.1) or triplets (A.2) as minimal self-containing structural units. For a discussion of factors causing the low number of such units see text. Scale bars represent 10μm.

ques at a receptor surface density of 0.80 mol %bPEG2000-DSPE [52]. Linker accumulation at 1.00 mol % bPEG2000-DSPE (Figure 4.D.1) indicates incomplete streptavidin coverage and is consistent with the experimental data shown in Figure 5b of [52]. Moreover, linker surface coverage is high enough for single vesicles to form several adhesion plaques (see arrows in Figure 4.D.1). Linkage-induced receptor accumulation [30, 54-56] is of particular interest in vesicle self-assembly due to its potential to self-terminate the assembly process by linker depletion and therefore to determine the coordination number of vesicles [29] in dependence of surface linker density [18, 57, 58]. Reducing the surface receptor density to 0.25 mol % bPEG2000-DSPE and increasing the number of distinct populations of complementary DNA strands decorating vesicles resulted in a small number of multi-vesicle structures of gradually increasing aggregate complexity (Figure 5). A detailed description of the experimental procedure is offered in [20]. However, the absolute number of linkers on the vesicle surface not only depends on the fraction of anchoring sites but also on the membrane area. In order to provide an effective self-terminating self-assembly process, vesicle size distribution may have to be monodisperse in addition to the constant surface linker density in future studies to ensure equal numbers of complementary linkers on the vesicle surfaces.

Multi-vesicle assemblies of predefined architecture (cp. Figure 5) may affect at least three distinct domains. (i) Analysis. Starting with a minimal system, the complexity of a bottom-up system may be gradually increased facilitating the understanding of the components and their interaction. Current bottom-up model systems in the analysis of biological processes are restricted to single unilamellar vesicles [3-5]. In this way, multi-vesicle assemblies provide an artificial bottom-up model system that allows to emulate and analyze natural cell-cell communication for instance (see (ii) for a scenario how to implement vesicle-vesicle communication in multi-vesicle assemblies). (ii) Synthesis. Externally triggered gating and channeling of confined cargo have already been described for a nanofluidic system which consists of two populations of nanometer-sized vesicles that are enclosed at random in a larger vesicle and consecutively release their attoliter volumes into the larger vesicle which serves as reaction vessel [13]. It has been proposed that communication within a DNA-mediated aggregate would become programmable and more reliable if its adhesion plaques differed in their phospholipid composition resulting in a multicompartment communication network of programmable architecture (see [48] for a discussion). Since this method could improve reliability, versatility and handling, such networks could potentially outperform current single or multicompartment bioreactors. (iii) Personalized medicine. Many therapeutic drugs have undesirable properties that constitute barriers in clinical drug application. Single vesicles are admittedly and successfully used as pharmaceutical carriers targeting active drugs to the site of action (see [10] for a comprehensive review). However, therapeutically effective multicompartment transports containing a pharmacologically inert prodrug [64] spatially separated from a converter enabling its transformation to an active drug molecule are currently unavailable. In this view, multi-vesicle assemblies such as those analyzed here offer such possibilities and thus represent a significant step in modern pharmacology (see [20] for a discussion).

MATERIALS AND METHODS

Vesicle formation (Figure 1 step 0)

Setups A, B, C: For a schematic illustration of the vesicle formation technique and technical terms used see [65] Figure 1. For modifications see [20, 21, 48]. The main aspects of modification recapitulated briefly: introduction of microplates to increase procedural manageability in laboratory experimentation and introduction of a density difference between the *inter*- and *intra*vesicular solutions to detach the vesicles from the interface between 'intermediate' and 'aqueous phase'. The 'emulsion phase' was prepared from sucrose or sucrose and sodium iodide (NaI), and the phospholipids POPC (2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine, Sigma-Aldrich, Buchs, Switzerland), bPEG2000-DSPE (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol)2000] (Ammonium Salt), Avanti Polar Lipids, Alabaster, AL), and cfPEG2000-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-

[poly(ethylene glycol)2000-N'-carboxyfluorescein] (ammonium salt), Avanti Polar Lipids, Alabaster, AL) that were solved in mineral oil (light, Sigma-Aldrich, Buchs, Switzerland) to a final concentration of 200 μM. POPC was purchased as powder and dissolved in chloroform to a total concentration of 5 mg/ml upon arrival. bPEG2000-DSPE and cfPEG2000-DSPE were purchased as 99% pure chloroform stock solutions (10 mg/ml) and used without further purification. The phospholipids dissolved in chloroform were kept at -20 °C until use. After chloroform evaporation (under vacuum, 60 min), addition of mineral oil, sonication (30 min), and overnight incubation at room temperature phospholipid solutions were used within several days. The phospholipid solutions were VP A1: 99 mol % POPC, 1 mol % cfPEG2000-DSPE, VP A2: 100 mol % POPC, VP B1: 90 mol % POPC, VP B2: 10 mol % bPEG2000-DSPE, and VP C1-C4: 99 mol % POPC, 1 mol % bPEG2000-DSPE. The same phospholipid solution was used to produce the 'intermediate phase' and the 'emulsion phase'. The 'aqueous phase' was prepared from 1000 mOsm glucose (setups A, B) and NaI (setup C, to a total osmolality of 1000 mOsm, for NaI concentrations see Figure 4). The water-in-oil emulsion of the 'emulsion phase' was equiosmolar to the 'aqueous phase' and was prepared in microtubes by adding 20 μl, 1000 mOsm sucrose (setups A, B) or sucrose/NaI (setup C, to a total osmolality of 1000 mOsm) solution to 1 ml phospholipid solution. The mixture was mechanically agitated, sonicated three times for five seconds and placed over the 'intermediate phase' (100 μl, placed over 100 μl 'aqueous phase'). After incubation (10 min, room temperature), centrifugation (1500 × g, 15 min, 4 °C) induced vesicle formation and pelletization in the centre of the well.

Vesicle decoration (Figure 1 steps 1, 2)

Setups B, C: Streptavidin (Sigma-Aldrich, Buchs, Switzerland) and streptavidin-AF488 (streptavidin, Alexa Fluor® 488 conjugate, Invitrogen, Basel, Switzerland) were dissolved in high quality water (Milli-Q, Millipore, Brussels, Belgium) upon arrival to a final concentration of 0.1 mg/ml. DNA single stranded oligonucleotides with biotin modification were synthesized, purified by HPLC, and dissolved (100 μM) by Sigma-Genosys (Buchs, Switzerland). The oligonucleotides sequences were biotin-TGTACGTCACAACTA-3' (biotin-α-3'-ssDNA), biotin-TAGTTGTGACGTACA-3' (biotin-α'-3'-ssDNA), and biotin-TGGAGGGCTCTTTCT-3' (biotin-β'-3'-ssDNA). The streptavidin solutions were either used directly (setup B), or streptavidins were redissolved (after evaporation) in glucose/NaI solution (setup C) to a final concentration of 333 nM, combined (1:1, v/v) with biotin-ssDNA solutions (666 nM), and individually incubated for 30 min at room temperature to provide monohomophilic oligonucleotide loading of streptavidin (streptavidin-AF488/biotin-α-3'-ssDNA, streptavidin/biotin-α'-3'-ssDNA, streptavidin/biotin-β'-3'-ssDNA). After aspirating the oil by vacuum, the vesicles were decorated with oligonucleotides. In setup B 90 μl of two vesicle populations were incubated (two hours, room temperature) with 10 μl streptavidin solution. In setup C, four vesicle populations were individually incubated (30 min, room temperature) with loaded streptavidin (two times streptavidin-AF488/biotin-α-3'-ssDNA, streptavidin/biotin-α'-3'-ssDNA, streptavidin/biotin-β'-3'-ssDNA). incubation (30 min). Excess streptavidin and oligonucleotides were removed by the following washing procedure (repeated three times): Pelletization (centrifugation at 1500 × g, 10 min, 4 °C), removal of supernatant (150 μl) and addition of 150 μl 'aqueous phase'.

Self-assembly of multi-vesicle structures (Figure 1 step 3)

Setup C: After pooling (streptavidin-AF488/biotin-α-3'-ssDNA & streptavidin/biotin-α'-3'-ssDNA, streptavidin-AF488/biotin-α-3'-ssDNA & streptavidin/biotin-β'-3'-ssDNA) and pelletization (centrifugation at 1500 × g, 10 min, 4 °C) of vesicle populations, vesicle aggregates were inspected by confocal laser scanning microscopy. Surface linker density is represented by the fluorescence signal of streptavidin-AF488. Inhomogeneities in the signal of fluorescently labeled streptavidin (accumulation *intra* and depletion *inter* contact areas) indicated formation of adhesion plaques. Presence of adhesion plaques qualified vesicle aggregates as assembled [30].

Surface modification

To prevent vesicles from adhering to surfaces, 96-

well microtiter plates U96 (Thermo Fisher Scientific, Langenselbold, Germany), microscope slides and cover glasses were specifically treated. Incubation steps (100 µl, 10 min, room temperature) were interrupted and followed by washing steps using deionized water of (i) microplates U96: 100 µl Repel Silane (GE Healthcare Europe GmbH, Otelfingen, Switzerland), 100 µl coating solution (10 mg/ml DNA from salmon sperm; Sigma-Aldrich, Buchs, Switzerland), 10 mg/ml BSA (in 1× PBS buffer; Roche Diagnostics GmbH, Mannheim); (ii) microtubes: 200 µl Repel Silane (vortexed several times during incubation to ensure total surface coverage); (iii) microscope slides and cover glasses: Repel Silane (total surface coverage). All surfaces were finally blown dry using compressed air. Observation chambers (area: 44 x 10 mm) for CLSM were made of Repel Silane treated microscope slides and cover glasses spaced to a distance of about 1 mm.

Microscopy

An inverted Leica Confocal DMR IRE2 SP2 microscope (Leica Lazer Technik, Heidelberg, Germany) equipped with a Zeiss HCX Apochromat 40.0×, 1.25-numerical-aperture oil immersion lens and Melles Griot argon laser (λ_{ex} = 488 nm) was used for Confocal Laser Scanning Microscopy. cfPEG2000-DSPE and streptavidin-AF488 were excited by the argon laser passing a TD 488/543/633 (setup A) or RSP 500 (setups B, C) excitation beam splitter. The epifluorescence was converted into a static beam by an x-y scanner device, passed a band-pass filter 508/31 (setup A), 507/24 (setup B), 509/20 (setup C) and was focused onto a photomultiplier (PMT fluorescence signal offset/HV settings: -1.7/712.2 (setup A), -11.3/793.7 (setup B), -92.1/672.0).

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CHAPTER 5

PROGRAMMABILITY OF MATTER: TO ALLOW FOR SPECIFICITY OF THE COMMUNICATION PROCESS

Publication Profile

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Publisher:	Springer-Verlag Berlin Heidelberg
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Book Series:	Lecture Notes in Artificial Intelligence
Pages:	126-135
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ISSN:	0302-9743
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Maik Hadorn Contributions:	Conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared the figures and tables.

Reviewer Comments

---------- review 1 ----------

OVERALL RATING:	0 (borderline paper)
REVIEWER'S CONFIDENCE:	0 (null)
originality:	3 (fair)
correctness:	3 (fair)
English:	5 (excellent)
relevance:	5 (excellent)

This paper is very clearly written but the technical details are outside my field of expertise.

---------- review 2 ----------

OVERALL RATING:	2 (accept)
REVIEWER'S CONFIDENCE:	0 (null)
originality:	4 (good)
correctness:	4 (good)
English:	5 (excellent)
relevance:	5 (excellent)

Seems relevant to its field, and the approach appears solid.

---------- review 3 ----------

OVERALL RATING:	3 (strong accept)
REVIEWER'S CONFIDENCE:	2 (medium)
originality:	4 (good)
correctness:	4 (good)
English:	5 (excellent)
relevance:	5 (excellent)

The paper is quite readable and provides context for a non-expert reader to appreciate. I judge its relevance to be well within the scope of ACAL's au-

dience, especially in its drawing parallels between 'real' biology and chemistry and their synthetic counterparts.

I should qualify my judgement by saying that I am far less expert in this area than the authors so I can not pick on any details of the originality and correctness. However, I go for strongly accept because, considering the specialised nature of the paper, it's still understandable for a less expert reader.

Regarding use of language: I like to discourage people from using 'Towards' in paper titles as it sounds wimpy when you actually have reasonably solid stuff to report as it is. Also the use of past tense in parts the abstract make the subject of the sentence seem like it's over or discontinued - a bad contrast with the 'towards' in the title!

Towards Tailored Communication Networks in Assemblies of Artificial Cells

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ABSTRACT. Living Technology is researching novel IT making strong use of programmable chemical systems. These chemical systems shall finally converge to artificial cells resulting in evolvable complex information systems. We focus on procedural manageability and information processing capabilities of such information systems. Here, we present a novel resource-saving formation, processing, and examination procedure to generate and handle single compartments representing preliminary stages of artificial cells. Its potential is exemplified by testing the influence of different glycerophospholipids on the stability of the compartments. We discuss how the procedure could be used both in evolutionary optimization of self-assembling amphiphilic systems and in engineering tailored communication networks enabling life-like information processing in multicompartment aggregates of programmable composition and spatial configuration.

KEYWORDS. Living Technology, self-assembly, programmability, glycerophospholipids, vesicles, multivesicular aggregates, adhesion plaque, phase transition

INTRODUCTION

Engineering Living Technology from non-living materials has attracted particular attention in minimal life and complex information systems. As part of the complex systems Future Emerging Technologies initiative, PACE (Programmable Artificial Cell Evolution) was researching novel Information Technology (IT) that makes strong use of life-like properties such as robustness, homeostasis, self-repair, self-assembly, modularity, self-organization, self-reproduction, genetic programmability, evolvability, complex systems design, and bootstrapping complexity. In this context, PACE has created the foundation for a new generation of embedded IT to build evolvable complex information systems using programmable chemical systems that converge to artificial cells [1]. Because experiments were realized both in the laboratory and in simulation, findings of *in vitro* and *in silico* experiments interacted and lead to essential additions to the evolutionary approach in design of laboratory experimentation [2].

According to the guidelines of PACE, the creation of simple forms of life from scratch in the laboratory should have pursued a bottom-up strategy choosing simple organic compounds of low molecular weight over highly evolved polypeptides. Complexity of an artificial cell featuring all aspects of a living system should have been achieved in an evolutionary process. It is hypothesized that a membrane partitioning internal constituents off the environment might have been one of the minimal requirements for living systems to arise [3, 4]. A lipid membrane represents a formidable barrier to the passage of

polar molecules. It organizes biological processes by compartmentalizing them. Compartmentalization enables segregation of specific chemical reactions for the purposes of increased biochemical efficiency by restricted dissemination and efficient storage of reaction products. This partitioning is not only realized between the cell and its environment, but it is even recapitulated within the cell. Vesicles, as an instance of minimality, feature an aqueous compartment partitioned off the surrounding by an impermeable lipid membrane. Like cellular membranes, vesicular membranes consist of amphiphilic phospholipids that link a hydrophilic "head" and a lipophilic "tail" (Figure 1). Suspended phospholipids can self-assemble to form closed, self-sealing solvent-filled vesicles that are bounded by a two-layered sheet (a bilayer) of 6 nm in width, with all of their tails pointing toward the center of the bilayer. This molecular arrangement excludes water from the center of the sheet and thereby eliminates unfavorable contacts between water and the lipophilic (= hydrophobic) tails. The lipid bilayer provides inherent self-repair characteristics due to lateral mobility of its phospholipids. Wide usage of artificial vesicles is found in analytics [5-9] and synthetics, where their applications include bioreactors [10-12], and drug delivery systems [13-17].

In the laboratory work, we focused on intrinsic information processing capabilities of vesicles and multivesicular assemblies. To achieve compartment-

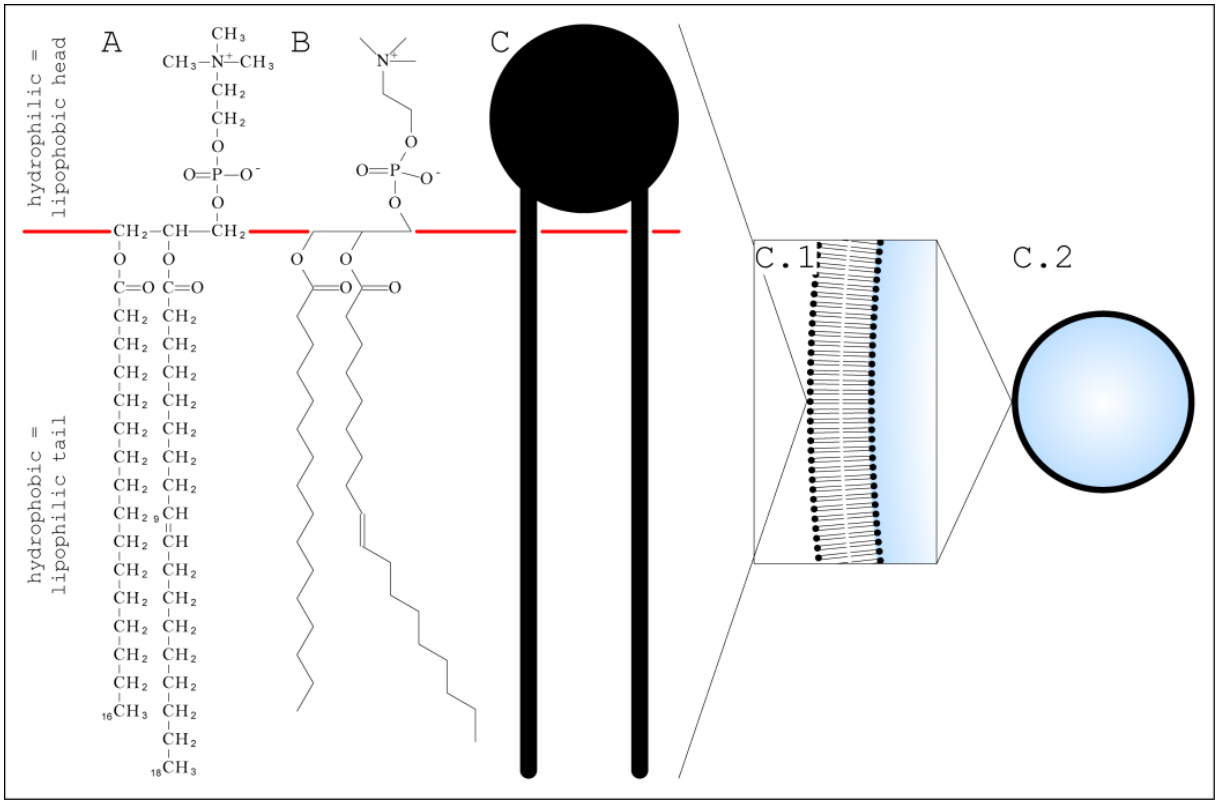

Fig. 1. Structure of glycerophospholipids and bilayer formation. The structure of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (PC(16:0/18:1(Δ9-Cis)) is represented (*A*) as a structural formula, (*B*) as a skeletal formula, and (*C*) as a schematic representation used throughout this publication. Glycerophospholipids are amphiphilic molecules with lipophilic hydrocarbon "tails" and hydrophilic "heads". In PC(16:0/18:1(Δ9-Cis) the headgroup is phosphatidylcholine; a saturated C_{16} palmitic acid (16:0) hydrocarbon chain and a monounsaturated C_{18} oleic acid (18:1) hydrocarbon chain occur at the C1 and C2 position of the glycerophospholipid and constitute the tail. The double bond (monounsaturation) occurs between the C9 and C10 atoms (Δ9) of the oleic acid, has cis configuration, and puts a rigid 30° bend in the hydrocarbon chain. (*C.1*) Phospholipids can form lipid bilayers (membranes) that partition an aqueous compartment off the surrounding medium. (*C.2*) In vesicles an *intra*vesicular fluid (light blue) is separated from the *inter*vesicular medium (white).

alization we developed methods to self-assemble multivesicular aggregates of programmable composition and spatial configuration composed of distinct vesicle populations that differ in membrane and *intra*vesicular fluid composition. The assembly process of multivesicular aggregates was based on the hybridization of biotinylated single-stranded DNA (ssDNA) with which the vesicles were doped. Doping was realized by anchoring biotinylated ssDNA to biotinylated phospholipids via streptavidin

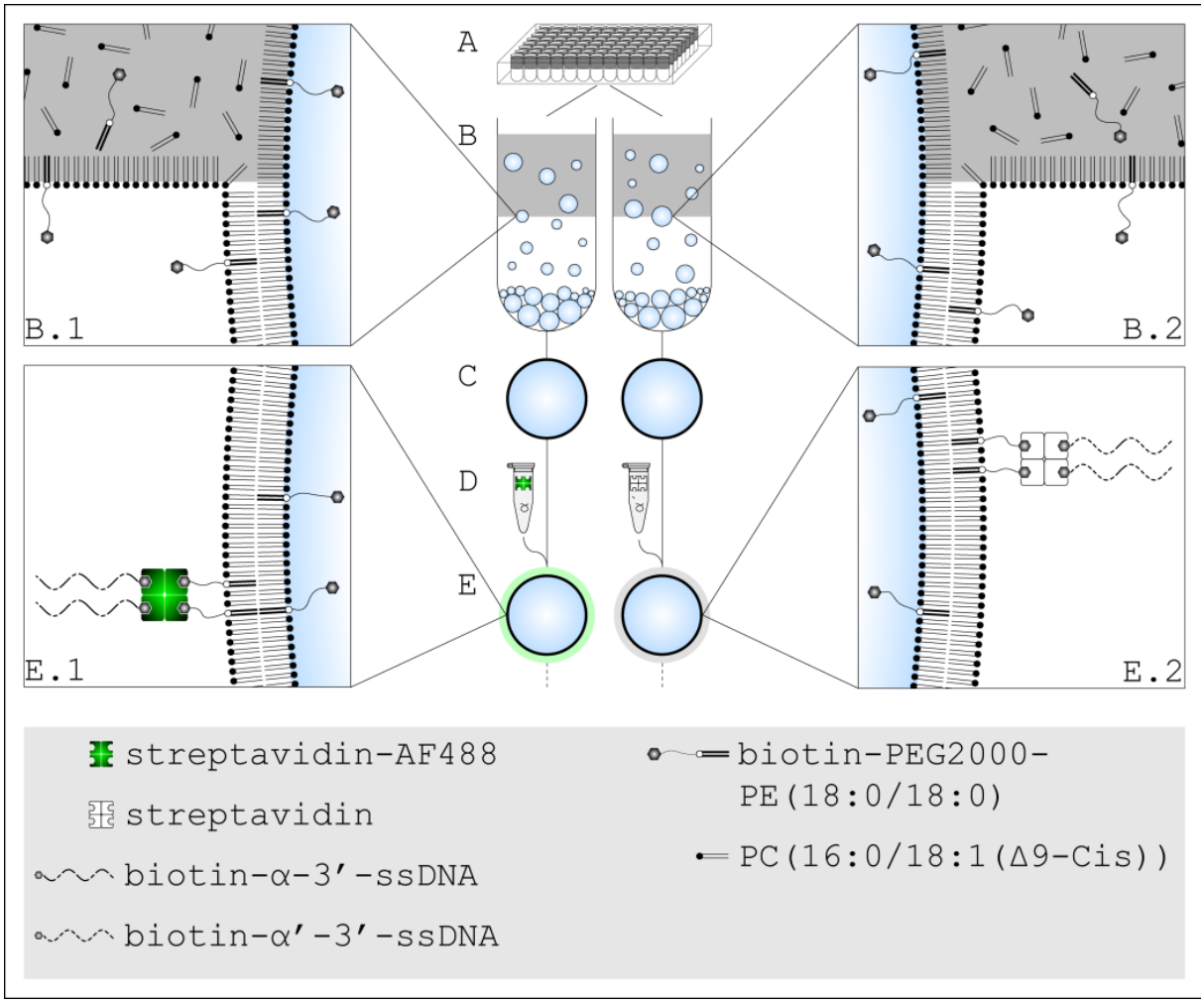

Fig. 2. Schematic representation of the parallel vesicle formation and membrane doping procedure. (*A*) Vesicles are produced in 96-well microtiter plates, providing parallel formation of up to 96 distinct vesicle populations. (*B*) The sample is composed of two parts: water droplets (light blue) in the oil phase (light gray) and the bottom aqueous phase (white), which finally hosts the vesicles. (*B.1*,*B.2*) Due to their amphiphilic character, glycerophospholipids (PC(16:0/18:1(Δ9-Cis)), biotin-PEG2000-PE(18:0/18:0)), solved in mineral oil, stabilize water-oil interfaces by forming monolayers. Two monolayers form a bilayer when a water droplet, induced by centrifugation, passes the interface. Glycerophospholipids are incorporated into the bilayer according to their percentage in the solution. Due to both the density difference of the *intra*- and *inter*vesicular fluid and the geometry of the microplate bottom, vesicles pelletize in the centre of the well (cp. *B*). (*C*) Vesicles remain in the same microtiter plate during formation and membrane doping. (*D*) Vesicle populations become distinct by incubating them with single stranded DNA (ssDNA) of different sequence (α: biotin-TGTACGTCACAACTA-3′, α′: biotin-TAGTTGTGACGTACA-3′) and streptavidin differing in fluorescence labeling (Alexa Fluor 488 conjugate (AF488) or unlabeled). (*E.1*,*E.2*) ssDNA covalently bound to biotin is non-covalently linked to phospholipid-grafted biotinylated polyethylene glycol tethers (biotin-PEG2000-Phosphoethanolamine) using streptavidin as cross-linking agent.

as a cross-linking agent (Figure 2). The potential of a programmable self-assembly of superstructures with significant attention to nanotechnological applications in the last decade. So far, cross-linkage based on DNA hybridization was proposed to induce self-assembly of complementary monohomophilic vesicles [5, 18] or hard sphere colloids [19-22], to induce programmable fusion of vesicles [5, 23], or to specifically link vesicles to surface supported membranes [5, 24-26]. By introducing a surface doping of distinct populations of ssDNA, as realized in the self-assembly of hard sphere colloids [27, 28], we provide n-arity to the assembly process. As a result, linkage of more than two distinct vesicle populations, as proposed by Chiruvolu *et al.* [29] and already realized for hard sphere colloids [30], becomes feasible.

Concerning procedural manageability in laboratory experimentation, we established a new protocol for *in vitro* vesicle formation and modification. It increases the versatility of the underlying vesicle formation method [11, 31, 32] by introducing microtiter plates and vesicle pelletization (Figure 2). The potential of the vesicle formation method that provides independent composition control of the *intra*- and *inter*vesicular fluid as well as of the inner and outer bilayer leaflet was exemplified by the production of asymmetric vesicles combining biocompatibility and mechanical endurance in asymmetric vesicles [31]. Here, we exemplify the advantages of the novel protocol by carrying out a high-throughput analysis of constituents affecting vesicle formation and stability. Thereby the effect of nine different glycerophospholipids on vesicle formation was tested. We discuss how this procedure could be used both in evolutionary optimization of self-assembling amphiphilic systems and to realize tailored communication networks in assemblies of artificial cells by programmable localization of glycerophospholipids within the vesicular membrane.

MATERIAL & METHODS

Major technical modifications of the vesicle formation protocol reported in Ref. [31] were: the introduction of (i) 96-well microtiter plates U96 to provide a high-throughput analysis and (ii) a density difference between *intra*- and *inter*vesicular solution to induce vesicle pelletization. For a description of the modified vesicle protocol see Figure 2. To analyze the effect of different glycerophospholipids on vesicle formation, data on vesicle yield of four times nine equimolar mixtures of 100%-m (m ∈ {100, 50, 10, 1}) PC(16:0/18:1(Δ9-Cis)) and m% PC(x:0/x:0) (x ∈ {12, 14, 16, 18, 24}), PC(y:1(Δ9-Cis)/y:1(Δ9-Cis)) (y ∈ {14, 16, 18}), or PC(24:1(Δ15-Cis)/24:1(Δ15-Cis)) were collected and compared to 100%-m PC(16:0/18:1(Δ9-Cis)) and m% mineral oil (solvent for all glycerophospholipids) as control.

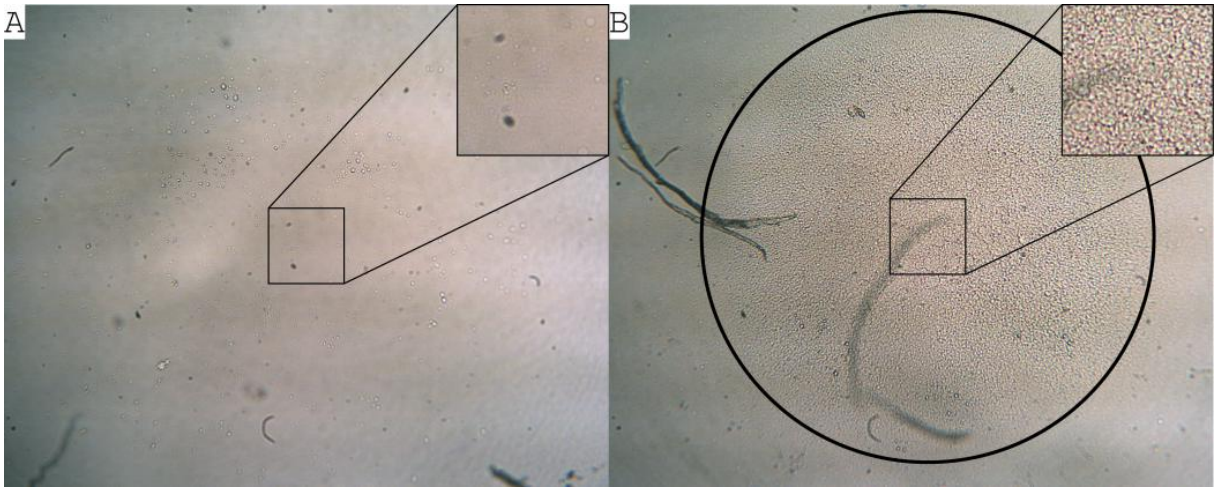

Fig. 3. Measurement of vesicle yield. (*A*) No vesicle pellet emerged for the mixture of 50% PC(24:1(Δ15-Cis)/24:1(Δ15-Cis)) and 50% PC(16:0/18:1(Δ9-Cis)). (*B*) The vesicle yield is expressed by length of circumference of the vesicle pellet (circle) emerged for the mixture 10% PC(24:1(Δ15-Cis)/24:1(Δ15-Cis)) and 90% PC(16:0/18:1(Δ9-Cis)). Fibers represent pollutants not affecting vesicular yield or handling.

Vesicle formation was performed in duplication. Length of circumference of the vesicle pellet is used as a measure of vesicle yield (Figure 3). Light-microscopy was performed using a Wild M40 inverted microscope equipped with a MikoOkular microscope camera. All camera settings were identical for the recordings. Confocal laser scanning microscopy was performed using an inverted Leica Confocal DMR IRE2 SP2 confocal laser scanning microscope.

RESULTS AND DISCUSSION

In the literature many examples of artificially produced vesicles are reported, whose membranes are composed of PC(16:0/18:1(Δ9-Cis)) (POPC) exclusively. To vary the intrinsic material properties of membranes we have to be able to alter their phospholipid content. In this study, we realized a high-throughput analysis of glycerophospholipids affecting vesicle formation. All glycerophospholipids differed in length and saturation of their hydrocarbon chains only. In the control experiment, vesicle yield was constant when POPC was present (Figure 4). Influence of the phospholipids tested at an admixture of one percent was marginal and will not be discussed. Intergroup comparisons (cp. Figure 4) of saturated and unsaturated glycerophospholipids of equal chain length revealed remarkable differences in vesicle yield. Whereas unsaturated glycerophospholipids of chain length up to 18 carbon atoms both could form vesicles by themselves and did not or just slightly affect the yield at 50 and 10 percent, saturated lipids seem to disturb vesicle formation down to 10 percent of admixture. For a chain length of 24 carbon atoms the situation is diametrical. A small amount of vesicles is found for solutions containing 100 percent saturated PC(24:0/24:0). Whereas vesicle yield is halved at 50 percent for this lipid, vesicle formation is inhibited totally by the unsaturated PC(24:1(Δ15-Cis)/24:1(Δ15-Cis)) up to an admixture of 10 percent (cp. Figure 3). Intragroup comparison within saturated or unsaturated glycerophospholipids reveals a decrease in vesicle yield depending on chain length (except for PC(24:0/24:0) at 100 percent). Only a limited number of glycerophospholipid is able to form vesicles on their own or in cooperation with POPC.

By providing parallelism in vesicle formation, processing and examination (cp. Figure 2), we not only increased the experimental throughput and the procedural manageability, but we lowered the costs and reduced the amount of contributory factors.

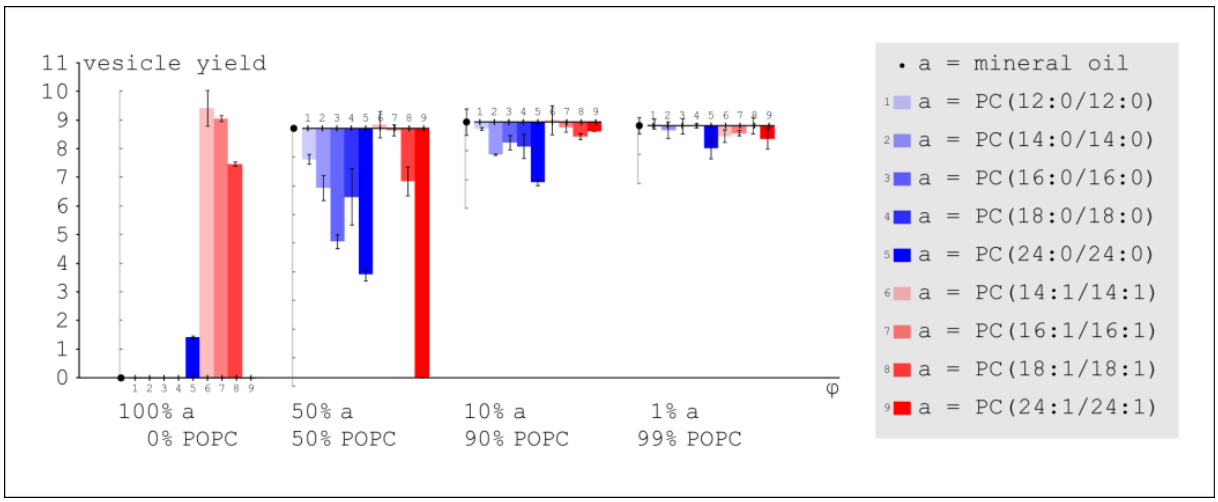

Fig. 4. Effect of different glycerophospholipids on vesicle formation. Vesicle yield of 100%-m (m ∈ {100, 50, 10, 1}) POPC = PC(16:0/18:1(Δ9-Cis)) and m% mineral oil serves as standard (XY (scatter) chart). Deviations from control for the glycerophospholipids tested are presented in absolute values as bar charts. Error bars indicate the standard deviation. The glycerophospholipids tested can be summarized according to the level of saturation of their hydrocarbon chains in two groups (saturated, PC(x:0/x:0) (x ∈ {12, 14, 16, 18, 24}); unsaturated, PC(y:1(Δ9-Cis)/y:1(Δ9-Cis)) (y ∈ {14, 16, 18}), PC(24:1(Δ15-Cis)/24:1(Δ15-Cis))).

This reduction of dimensionality and the parallelism in vesicle formation provided by the novel vesicle formation method presented herein may prove to be useful in evolutionary design of experiments by shortening the search toward the optimality region of the search space [2]. The application of microtiter plates may further enable the automatization in vesicle formation.

By self-assembling multivesicular aggregates of programmable composition and spatial configuration, composed of vesicles differing in membrane and *intra*vesicular fluid composition, an inhomogeneous distribution of information carriers is achieved (Figure 5). To build complex information systems, the exchange of information carriers between the compartments themselves and/or between the environment and the compartments has to be realized. Biological membranes contain membrane proteins that were evolutionary optimized for catalyzing numerous chemical reactions, mediating the flow of nutrients and wastes, as well as participating in communication across the membrane. The use of such membrane proteins in information exchange was excluded by the guidelines of PACE. Thus, information processing capabilities of multivesicular aggregates had to be realized by exploiting intrinsic material properties of the phospholipid membrane. A key property of the lipid membrane is its phase [33]. Lipid bilayers can undergo phase transitions in which they become a gel-like solid and therefore lose their fluidity. The state depends on temperature; the transition temperature of a bilayer increases with the chain length and the degree of saturation of its fatty acid residues. The phase transitions are triggered externally by chang-

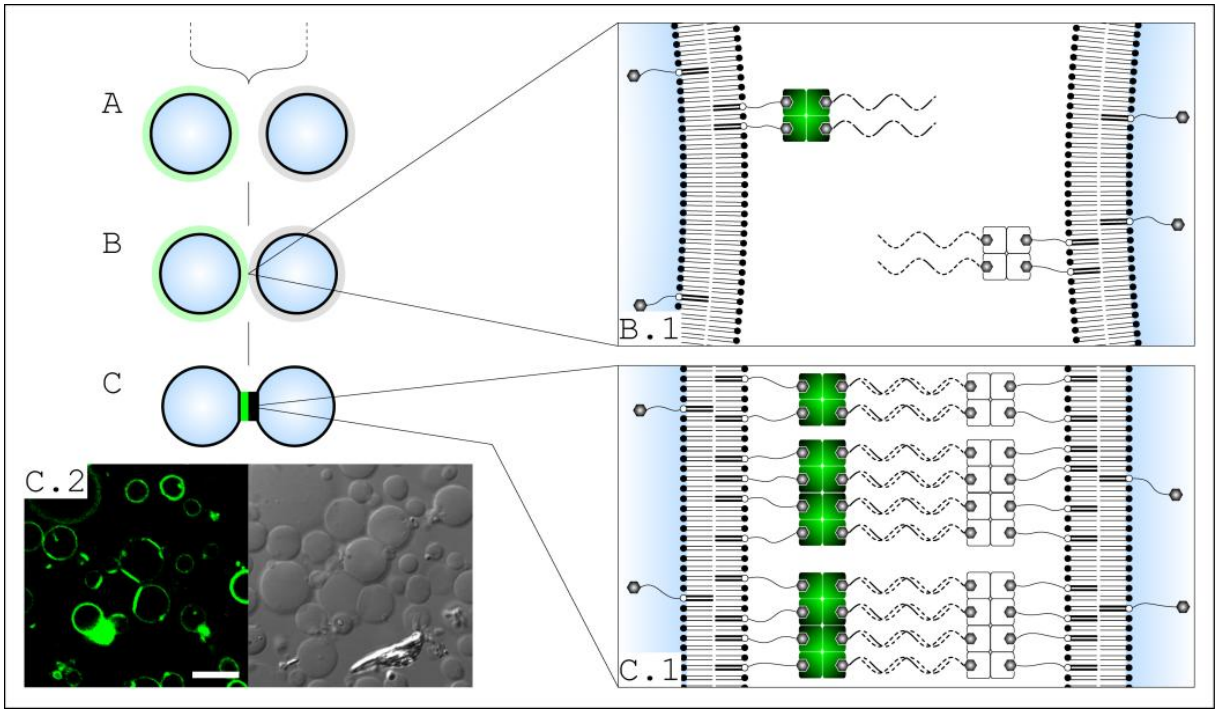

Fig. 5. Schematic representation of the self-assembly process and micrographs of adhesion plaques. (*A*) For vesicle formation, membrane doping and illustration symbols see Figure 2.Two distinct vesicle populations are merged (brace). (*B*) The lateral distribution of linkers in the lipid membrane is homogeneous. (*B.1*) Vesicles doped with complementary single stranded DNA come into contact. (*C*) Hybridization of DNA strands results in double stranded DNA and induces the assembly process. Due to their lateral mobility, linkers accumulate in the contact zone forming an adhesion plaque – the lateral distribution of linkers in the outer leaflet becomes inhomogeneous. (*C.1*) Biotinylated phospholipids (biotin-PEG2000-PE(18:0/18:0)) colocalize with the linkers. Even though the lateral distribution of phospholipids in the inner leaflet is not affected, the membrane composition *intra*-adhesion-plaque (by accumulation) and *inter*-adhesion-plaque (by depletion) becomes different. (*C.2*) CLSM (confocal laser scanning microscope) and DIC (differential interference contrast) micrograph of a vesicular aggregate. Accumulation and depletion of linkers are clearly visible in the CLSM micrograph. Scale bar represents 10μm.

ing temperature; a fact exploited in exchanging reactants between the surrounding medium and a vesicular compartment setting up consecutive enzymatic reactions in a single container [34]. A selective exchange of information between compartments of a multivesicular aggregate relying on inherent material properties of phospholipid membranes has not been demonstrated so far. We recognized that the formation of adhesion plaques [35, 36] triggered by the aggregation process results in an inhomogeneous distribution of phospholipids in the lateral dimension of the membrane (Figure 5). This inhomogeneity in phospholipid distribution causes a difference in phase transition characteristics of membrane portions *intra-* and *inter-*adhesion-plaque. Selective opening of communication channels between the compartments, whereas the membrane portions *inter-*adhesion-plaques are still impermeable, becomes conceivable. By using membrane anchors of different phospholipid composition the adhesion plaques would differ in phase transition characteristics among each other enabling programmable and triggerable communication networks in multivesicular aggregates of programmable composition and spatial configuration.

Realization of such tailored communication networks is hindered by restrictions in commercial availability of lipid anchors to dope vesicular surfaces with ssDNA. The high-throughput analysis presented herein allows for identification of lipid candidates not affecting vesicle formation and differing in phase transition characteristics. In a next step the head group of these candidates could be covalently linked to biotinylated PEG tethers therefore providing anchors for biotinylated ssDNA (via (strept-)avidin).

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CHAPTER 6

INFORMATION PROCESSING: TO FLOW INFORMATION IN SELF-ASSEMBLED COMPARTMENTS

Publication Profile	
Title:	Development of an externally triggerable multicompartment communication network of programmable architecture and evaluation of its potential in analytics, synthetics, and medical applications
Year:	submitted: October 1, 2009; approved: November 24, 2009
Authors:	Hadorn M
Publication Type:	Proposal for a 'fellowship for prospective researches'

Development of an Externally Triggerable Multicompartment Communication Network of Programmable Architecture and Evaluation of Its Potential in Analytics, Synthetics, and Medical Applications

Maik HADORN,

Artificial Intelligence Laboratory, Department of Informatics, University of Zurich, Zurich, Switzerland

Abstract. Engineering living from non-living materials has attracted particular attention in minimal life and complex information systems where programmable, artificial, cell-like compartments are intended to converge to living cells. Whereas single cell-like compartments already serve as research model in the analysis of natural cells, in the synthesis of bioreactors, and in medical applications, the potential offered by their aggregates is exploited only marginally. In this context, the applicant succeeded in establishing a DNA-mediated self-assembly process. The high specificity of binding between complementary sequences and the digital nature of DNA base coding resulted in programmable composition and spatial arrangement of aggregates of cell-like compartments. Using insights from these former studies, a communication between assembled cell-like compartments is proposed herein. Thus, externally triggerable multicompartment communication networks (MCCNs) of programmable architecture that are applicable in the analysis of natural cells and that will outperform current bioreactor and drug delivery systems shall be implemented in this project.

Relevance of the Fellowship

Living Technology is promoted by the European Commission through funding of current and completed research projects (PACE[1] [1], MATCHIT[2]). In this scope, the University of Southern Denmark (SDU, Odense, Denmark) as one of the founding institutions of the European Center for Living Technology[3] (ECLT, Venice, Italy) has developed into the leading center of Living Technologies in Western Europe. Whereas the ECLT is mainly an international meeting point, the Mærsk Mc-Kinney Møller Institute (MMMI) of SDU is dedicated to the transfer of results from basic research in cell structure, communication, and development to the creation of new technologies for the productive sector and to transfer these technologies to various sectors such as pharmaceutics, medicine and environment. The Mærsk Mc-Kinney Møller Institute for Production Technology was established in 1997 as part of the Faculty of Science of the University of Southern Denmark. In 1999, the Institute moved into new premises donated by the A.P. Møller and Chastine Mc-Kinney Møllers Foundation. The aim of the Institute is to become a highly technological, internationally recognized centre of excellence, where academia and industry in close collaboration develop new technologies. A close in-house collaboration of MMMI and the Center for Fundamental Living Technology (FLinT, SDU) provides access to a variety of experimental, computational, and theoretical techniques for the synthesis and analysis of energetics, self-assembly, self-replication, self-organization, evolution, motility, and simple intelligence, inorganic-, organic-, bio-, and supramolecular chemistry, multiscale molecular- and microfludics modeling, and dynamical systems and stochastic processes. As participants of MATCHIT, MMMI and FLinT closely collaborate and intend to consolidate their leading position in Living Technology and to focus on a new generation of technologies which embody the essential properties of life, such as self-organization,

[1] Programmable Artificial Cell Evolution. Integrated project, European Union EU-IST-FP6-FET-002035, duration: 04/04-06/08. The Artificial Intelligence Laboratory (Head Prof. Rolf Pfeifer, Department of Informatics, University of Zurich) was participant of this project.

[2] Matrix for Chemical Information Technology. Small or medium scale focused research project, European Union. Passed the negotiations in Brussels, Sept. 3, 2009. Start date: 02/10.

[3] In addition to SDU, the University of Zurich was also one of the founding institutions of ECLT.

adaptability, and capacity to evolve and react to environmental stimuli.

The proposed project perfectly matches this focus and will enable the applicant both to intensify his collaboration with SDU and to extend his previous research on Living Technology performed at the Artificial Intelligence Laboratory (AILab) of Professor Rolf Pfeifer (Department of Informatics, University of Zurich (UZH), Switzerland). Maik Hadorn studied and graduated in Biology at UZH. In the scope of the projects EES[4] and PACE and in close collaboration with the MMMI, the applicant studied novel Information Technology making strong use of life-like properties such as robustness, self-repair, self-assembly, modularity, self-organization, genetic programmability, and evolvability. Wet laboratory experiments were performed at the Molecular Physiology Laboratory of Professor Enrico Martinoia (Institute of Plant Biology, UZH). Thus, Maik Hadorn is trained in biological research and concepts, skillful in Living Technology, and well experienced both in *in vitro* and *in silico* laboratory work. The applicant feels confident that SDU is the ideal scientific environment for his personal and professional growth. By supporting his aim to transfer results from basic research to future industrial applications such as modular robots, personalized drug delivery systems, and bioreactors all composed of programmable cell-like self-assembling entities, the stay at SDU will provide access to a variety of technical equipment to analyze his experiments and allow him to profit from present academic expertise in Living Technology.

In addition to the participation of the AILab in the project PACE, research at the Swiss Federal Institute of Technology (Zurich, Switzerland) and the School of Life Sciences and Facility Management (Zurich University of Applied Sciences, Waedenswil, Switzerland) are related to Living Technology. Nevertheless, the end of the project PACE caused a weakening in research of Living Technology in Switzerland. In the follow-up project MATCHIT no Swiss institution is participating. Informal meetings initiated by the AILab with members of the Swiss Federal Institute of Technology and the School of Life Sciences and Facility Management revealed a particular interest both from industrial and scientific perspective in establishing a Swiss Living Technology Competence Network as a long-term goal. The applicant shares this aim and would like to contribute to embed this novel discipline with potential applications in the medical, material, information, energy, and environmental sciences in the university system of Switzerland.

[4] Embryogenic Evolution: From Simulations to Robotic Applications. Swiss National Foundation 200020-118127. Doctoral thesis of Maik Hadorn.

Project Description

Aim

Engineering living from non-living materials has attracted particular attention in minimal life and complex information systems where programmable, artificial, cell-like compartments are intended to converge to living cells. Whereas single cell-like compartments already serve as research model in the analysis of natural cells, in the synthesis of bioreactors, and in medical applications, the potential offered by their aggregates is exploited only marginally. In this context, the applicant succeeded in establishing a DNA-mediated self-assembly process. The high specificity of binding between complementary sequences and the digital nature of DNA base coding resulted in programmable composition and spatial arrangement of aggregates of cell-like compartments. Using insights from these former studies, a communication between assembled cell-like compartments is proposed herein. Thus, an externally triggerable multicompartment communication networks (MCCNs) of programmable architecture that is applicable in the analysis of natural cells and that will outperform current bioreactor and drug delivery systems shall be implemented in this project.

State-of-the-Art

Tethered Multicompartment Systems

Artificial vesicles, as entities of cell-like compartments, feature an aqueous compartment partitioned off an aqueous environment by a closed lipid membrane that is nearly impermeable for hydrophilic substances. Like cellular membranes, vesicular membranes consist of amphiphilic phospholipids that link a hydrophilic head and a lipophilic tail. The

lipid bilayer organizes processes by compartmentalizing them and provides inherent self-repair characteristics due to lateral mobility of its phospholipids [2]. Detailed insights into the underlying molecular mechanisms are impeded in many cases by the excess of components in natural cells. As a result of the analogy to natural systems and the compositional simplicity, artificial vesicles are the most studied systems among biomimetic structures [3] providing a bottom-up procedure in the analysis of biological processes [4-6]. In addition, vesicles are applied in synthetics where they are used as minilaboratories to study confined chemical reactions under biologically relevant conditions [7] and bioreactors [8-10]. Their ability of controlled confinement, transport, and manipulation of chemical cargo is used in vesicular drug delivery systems [11-13]. In general, single unilamellar vesicles apply to analytic, synthetic, and medical applications. Multicompartment systems on the other hand offer a division of different membrane functions (confinement, biocompatibility, cargo release, targeting, protection) among membranes differing in composition and dimension. Specific chemical reactions can be segregated for the purposes of increased controllability, observability, stability, and biochemical efficiency by restricted dissemination and efficient storage of reactants, and/or reaction products. Thus, tethered multivesicular systems are realized in bioreactor [14, 15], and cosmetic applications [16] and are proposed as multicomponent or multifunctional drug delivery systems [17-20]. The programmable self-assembly of superstructures composed of *n* distinct entities with high degrees of complexity [21] has attracted significant attention in nanotechnological applications [22-25].

In close collaboration with SDU, the applicant implemented DNA-mediated linkage of more than two distinct vesicle populations providing the first implementation of real *multi*component or *multi*functional systems of programmable composition and spatial arrangement [26]. In contrast to the handmade reactors and networks [15, 27, 28], self-assembly offers cheap and large-scale production of MCCN bioreactors.

Externally Triggerable Communication in Multicompartment Systems

Lipid bilayers can exist both in solid-like (gel) states, in which the lipids form a tightly packed ordered array that undergoes little motion, and in a more loosely packed and liquid-like, disordered fluid state, in which the lipids undergo considerable motion. The temperature at which the lipids within a bilayer undergo the transition from the ordered to the fluid state is called the transition temperature. Membranes varying in saturation and/or length of the fatty acid residues of their phospholipids differ in this transition temperature [29]. Only if ordered and fluid phases coexist on the same membrane (i.e. during phase transition), membranes become permeable [30, 31]. The difference in the phase transition temperature of different vesicles enclosed in a single reaction vessel was exploited in setting up consecutive enzymatic reactions in a single compartment, whereby the release of cargo took place across the whole vesicle surface [14]. In the proposer's previous work [26, 32, 33], vesicle membranes were composed of two populations of phospholipids: One providing anchorage of single stranded DNA (ssDNA) to the vesicle surface while the other providing the basic structure of the vesicle. Because ssDNA is linked to a phospholipid, the lateral mobility of the phospholipids in the lipid membrane is transmitted to ssDNA. Thus, DNA hybridization induces vesicle-vesicle linkage and results in a linker accumulation *intra* contact areas resulting in 'adhesion plaques' (cp. [19, 34-40]) as well as in a linker depletion *inter* contact areas [26, 32, 33]. Since the phospholipid anchors co-accumulate with the linkers, the two phospholipid populations become laterally separated in the vesicle membrane as a result of the self-assembly process.

Hypothesis

Phospholipid membranes of a particular composition confining cell-like compartments are characterized by an exact phase transition temperature. At this temperature, membranes become permeable and release entrapped cargos along their concentration gradients. The DNA-mediated self-assembly process based on hybridization of single stranded DNA induces a lateral accumulation of particular phospholipids at adhesion sites and therefore an

inhomogeneous distribution of phospholipids in the membrane. This inhomogeneity results in different phase transition temperatures in the lateral dimension of the membrane. It is assumed that at a certain temperature the membrane will be permeable just at the adhesion sites, whereas the residual membrane remains impermeable (Hypothesis 1). This would permit externally triggered and directed communication between adjacent cell-like compartments. Different phospholipids that provide anchorage to a multitude of different DNA single strands can be integrated into the vesicle membrane. If DNA populations are distinct in their phospholipid anchorage, a sequence specific accumulation of phospholipids at adhesion sites results when a compartment is adjacent to two or more non-corresponding compartments. Since the adhesion sites would therefore differ among themselves and from the residual membrane, it is postulated that communication pathways in a MCCN of programmable architecture becomes externally controllable (Hypothesis 2). If channeling and gating of communication in MCCNs are externally controllable, MCCNs will outperform current bioreactor and drug delivery systems (Hypothesis 3).

Scenario

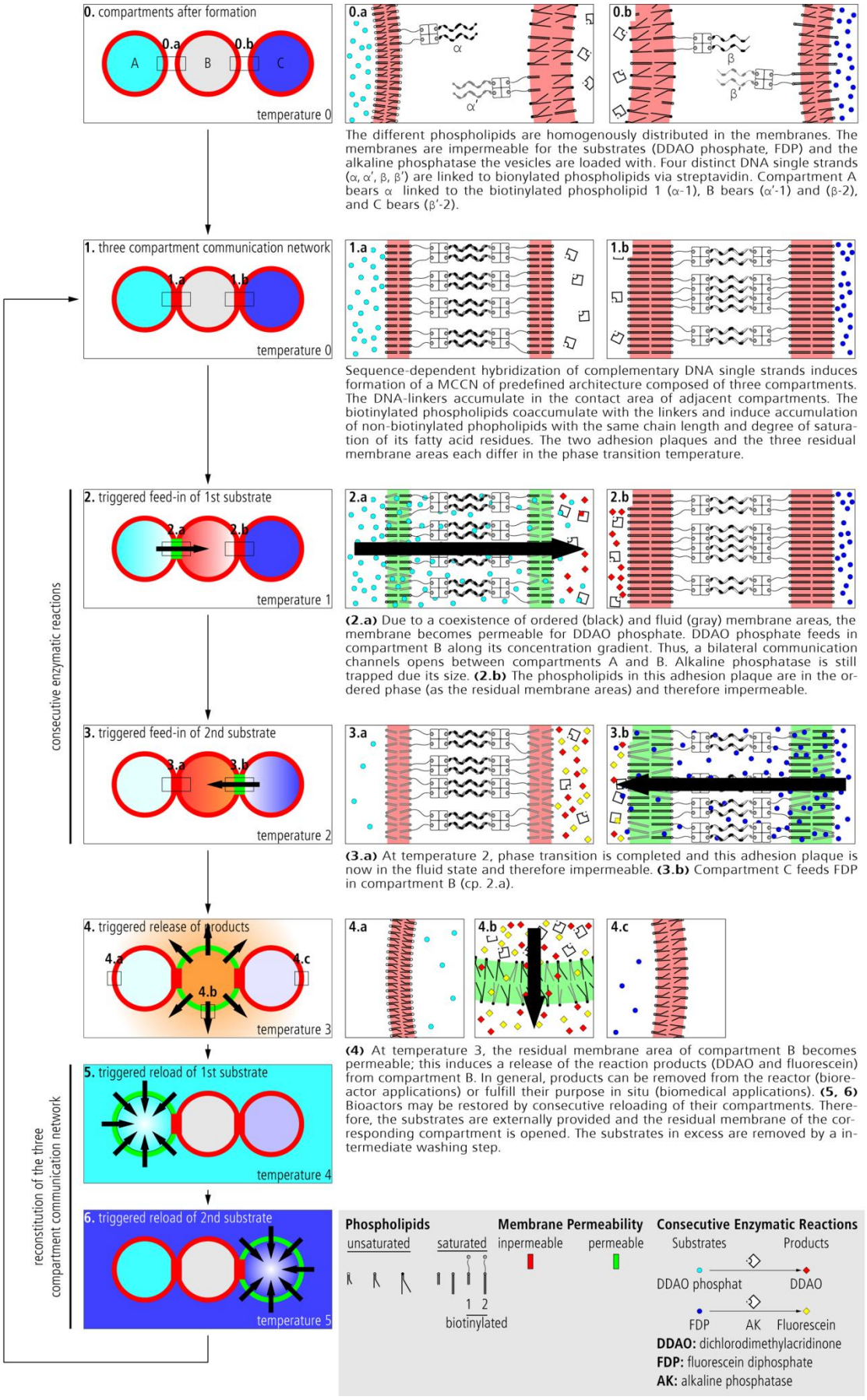
Three distinct vesicle populations are produced that differ in the phospholipids incorporated in their membrane. One vesicle population is loaded with an enzyme catalyzing two different substrates, individually incorporated in the two other vesicle populations (Fig.1.0). These vesicle populations become adjacent by a DNA-mediated self-assembly process [26, 32, 33] resulting in basic MCCNs of predefined architecture each composed of three vesicles (Fig.1.1). The DNA-mediated self-assembly process induces an inhomogeneous distribution of phospholipids. The two emerging adhesion plaques and the three residual membrane areas each differ in the phase transition temperature. A change of temperature ($t_0 \rightarrow t_1$) induces a phase-transition of one adjacent membrane area (Fig.1.2.a), opens a bilateral communication channel via a small intermembrane gap whereas the all other membrane areas are still impermeable (cp. Fig.1.2.b), and enables a feed-in of the first substrate. The same procedure is applied at a different temperature (t_2) for the triggered feed-in of the second substrate (Fig.1.3). The distinct phase transition temperatures of the residual membrane areas are exploited in the triggered release of reaction products (at t_3, Fig.1.4), and the individual reload (at t_4 and t_5) of the two vesicle populations incorporating the reaction substrates (Figs.1.5/1.6). The regeneration of the initial state and the potential reuse of the MCCN represent one of the main advantages of the proposed system. Due to the lack of externally controllable communication channels, current bioreactors that implement consecutive reactions (cp. [14]) are disposable after substrates are consumed completely.

Relevance

Programmable MCCNs of predefined architecture have not been implemented so far and will affect at least three distinct domains. (i) Analysis. Starting with a minimal system, the complexity of bottom-up systems is increased gradually facilitating the understanding of the components and their interaction. Thus, MCCNs will provide an artificial bottom-up model system to emulate and analyze natural cell-cell communication. (ii) Synthesis. Gating and channeling of confined cargo will be externally controllable in MCCNs. Thus, consecutive reactions may be implemented outperforming current bioreactors in versatility and productivity. (iii) Medical applications. Transport of long-lived, inactive pre-drugs and their externally triggered conversion to short-lived, active drugs right at the target site will become possible when using MCCNs.

The network architecture is programmable by the DNA-mediated self-assembly (see [33]), whereas gating and channeling in the communication network is externally controllable by temperature changes. Thus, paracrine and endocrine signaling pathways that play important roles in development, metabolism, and growth may be analyzed in an artificial model system and exploited in bioreactor, pharmaceutical, information processing, and material science applications. Consecutive compartmentalized chemical reactions may result in an out-

Figure 1 (opposite). *Schematic representation of the implementation of two consecutive enzymatic reactions in communication networks made of three compartments.*


0. compartments after formation
A
0.a
B
0.b
C
temperature 0
0.a
α
α'
0.b
β
β'
The different phospholipids are homogenously distributed in the membranes. The membranes are impermeable for the substrates (DDAO phosphate, FDP) and the alkaline phosphatase the vesicles are loaded with. Four distinct DNA single strands (α, α', β, β') are linked to bionylated phospholipids via streptavidin. Compartment A bears α linked to the biotinylated phospholipid 1 (α-1), B bears (α'-1) and (β-2), and C bears (β'-2).
1. three compartment communication network
1.a
1.b
temperature 0
1.a
1.b
Sequence-dependent hybridization of complementary DNA single strands induces formation of a MCCN of predefined architecture composed of three compartments. The DNA-linkers accumulate in the contact area of adjacent compartments. The biotinylated phospholipids coaccumulate with the linkers and induce accumulation of non-biotinylated phopholipids with the same chain length and degree of saturation of its fatty acid residues. The two adhesion plaques and the three residual membrane areas each differ in the phase transition temperature.
2. triggered feed-in of 1st substrate
2.a
2.b
temperature 1
2.a
2.b
(2.a) Due to a coexistence of ordered (black) and fluid (gray) membrane areas, the membrane becomes permeable for DDAO phosphate. DDAO phosphate feeds in compartment B along its concentration gradient. Thus, a bilateral communication channels opens between compartments A and B. Alkaline phosphatase is still trapped due its size. (2.b) The phospholipids in this adhesion plaque are in the ordered phase (as the residual membrane areas) and therefore impermeable.
consecutive enzymatic reactions
3. triggered feed-in of 2nd substrate
3.a
3.b
temperature 2
3.a
3.b
(3.a) At temperature 2, phase transition is completed and this adhesion plaque is now in the fluid state and therefore impermeable. (3.b) Compartment C feeds FDP in compartment B (cp. 2.a).
4. triggered release of products
4.a
4.b
4.c
temperature 3
4.a
4.b
4.c
(4) At temperature 3, the residual membrane area of compartment B becomes permeable; this induces a release of the reaction products (DDAO and fluorescein) from compartment B. In general, products can be removed from the reactor (bioreactor applications) or fulfill their purpose in situ (biomedical applications). (5, 6) Bioactors may be restored by consecutive reloading of their compartments. Therefore, the substrates are externally provided and the residual membrane of the corresponding compartment is opened. The substrates in excess are removed by a intermediate washing step.
5. triggered reload of 1st substrate
temperature 4
reconstitution of the three compartment communication network
6. triggered reload of 2nd substrate
temperature 5
Phospholipids
unsaturated
saturated
1 2
biotinylated
Membrane Permeability
inpermeable
permeable
Consecutive Enzymatic Reactions
Substrates
Products
DDAO phosphat
DDAO
FDP
AK
Fluorescein
DDAO: dichlorodimethylacridinone
FDP: fluorescein diphosphate
AK: alkaline phosphatase

performance of MCCN-based bioreactors compared to current single-compartment-based production facilities. Further, confinement of long-lived, inactive pre-drugs and their *in situ* conversion to short-lived, active drugs – by opening a communication channel between adjacent vesicles – as well as their release – by opening membrane of the reaction vessel – may be implemented by using MCCNs. Targetability of pharmaceutical nanocarriers such as liposomes [41] and local application of external heat at target sites [42, 43] are well developed. Thus, MCCNs could be traced to or passively accumulate at target sites [12] and produce and release active drugs only at target sites. This reduces both the amount of drugs applied and harmful side-effects.

Method

Work Package 1: Analysis

WP1a: Vesicle stability in dependence of the phospholipids composition (project month (PM) 1-3). To test the hypotheses formulated above, phospholipids anchoring ssDNA and the phospholipids providing the basic structure have to differ in chain length and saturation level of their fatty acid residues. We already studied vesicle production and lysis rate in dependence of the phospholipids used [32]. In WP1a these experiments are continued to evaluate different phospholipid candidates.

WP1b: Temperature dependent permeability of vesicles (PM 3-6). Alkaline phosphatase (AP) is encapsulated in vesicle using current vesicle formation methods [9, 44] whereas fluorescein diphosphate (FDP) is provided in the surrounding medium. Vesicles are made of phospholipids that passed WP1a. AP catalyzes the conversion of non-fluorescent FDP to fluorescein. Fluorescence intensity of fluorescein as function of FDP concentration and temperature (cp. [14]) is analyzed.

Milestone 1 *(PM 6)*: Identification of phospholipids least interfering with vesicle production and with broadest possible range of phase transitions temperatures. *MS1 failed:* Vesicle formation procedure is adjusted (see [15] for a list of production methods). *MS1 passed:* Identified phospholipids are purchased as custom-synthesized biotinylated PEG tether grafted phospholipid from Avanti Polar Lipids (Alabaster, AL).

Work Package 2: Synthesis

WP2a: *Implementation of communication networks made of two compartments (PM 7-12).* DNA-mediated linkage of two vesicle populations (VPs) is already established [26, 32, 33]. Since accumulation of linkers results in self-termination of the assembly process (data unpublished), the size of the aggregates is adjusted by variations in surface linker density. To analyze vesicle-vesicle interchange of cargo in dependence of temperature, one VP will enclose AP, the other FDP. Different biotinylated PEG tether grafted phospholipids will be used to anchor ssDNA to the vesicular surface. Vesicle-vesicle communication will be analyzed by temperature dependent localization of the fluorescence signal. If differences in membrane composition of adhesion plaques from the residual membrane areas are too low for a definite channeling, non-biotinylated entities of the phospholipids are admixed (cp. Fig. 1). It is supposed that non-biotinylated phospholipids will coaccumulate with biotinylated phospholipids of the same chain length and degree of saturation of their fatty acid residues (cp. raft formation [45]). Thus, phospholipid composition of the adhesion plaques would become more distinct from the residual membrane areas.

Milestone 2 *(PM 12)*: Verification of the hypothesis 1.

MS2 failed: Reformulation of the project. Multicompartment systems will be produced and encapsulated (as discussed in [26]). The encapsulating compartment serves as reactor vessel of chemical cargo consecutively released (cp. [14]).

MS2 passed: **WP2b:** *Implementation of consecutive enzymatic reactions in communication networks made of three compartments (PM 13-16)*. The consecutive enzymatic reactions are derived from [14]. For a detailed schematic representation see Figure 1. One of the three VPs is doped with two distinct ssDNA addresses linking it to both other vesicle populations. This central compartment contains the AP, one contains FDP and the other DDAO phosphate. An increase of temperature will trigger the

consecutive transfer of DDAO phosphate and FDP into the central compartment, where they become fluorescently active due to the enzymatic conversion to DDAO and fluorescein. Production and localization of the particular fluorescent products differing in their emission spectrum are analyzed.

Milestone 3 *(PM 16)*: Implementation of consecutive enzymatic reactions in externally triggerable MCCNs.

Work Package 3: Applications

MS3 passed: **WP3a:** *Peer-reviewed publications and communication of results to industry (PM 16-18).* Results are published and patented, if needed, and communicated to potential industrial partners to develop the architecture and communication setup of a MCCN for demonstration purposes.

WP3b: *Implementation of WP3a's MCCN (PM 19-22).*

WP3c: *Peer-reviewed publications and communication of results of WP3b (PM 23-24).*

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CHAPTER 7

FUNCTIONALITY: TO ALLOW FOR *IN VIVO* DRUG SYNTHESIS JUST-IN-TIME-AND-PLACE

Publication Profile

Title:	Encapsulated Multi-vesicle Assemblies of Programmable Architecture: Towards Personalized Healthcare
Year:	2010
Authors:	Hadorn M, Eggenberger Hotz P
Editors:	TBA
Publication Type:	Book Chapter
Publisher:	Springer-Verlag Berlin Heidelberg
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Book Series:	Communications in Computer and Information Science
Pages:	TBA
Volume:	TBA
ISSN:	TBA
Conference:	3rd International Joint Conference on Biomedical Engineering Systems and Technologies (BIOSTEC), Jan 20-23, 2010, Valencia, Spain
Maik Hadorn Contributions:	Conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared the figures and tables.

Reviewer Comments

Scale: 1: lowest value; 6: highest value

---------- review 1 ----------

Abstract and Introduction are adequate? **Yes**

Conclusions/Future Work are convincing? **Yes**

Figures are Adequate? in number and quality **Yes**

Improve critical discussion ? **No**

Improve English? **No**

Needs comparative evaluation? **No**

Needs more experimental results? **No**

Originality: Newness of the ideas expressed **5**

Overall rating **5**

Paper formatting needs adjustment? **No**

Presentation: Structure/Length/English **5**

References are up-to-date and appropriate? **Yes**

Relevance: Paper fits one or more of the topic areas? **5**

Significance: Is the problem worth the given attention? **5**

Technical Quality: Theoretical soundness/methodology **5**

Comment: Authors should describe future research plans.

---------- review 2 ----------

Abstract and Introduction are adequate?
Yes

Conclusions/Future Work are convincing?
Yes

Figures are Adequate? in number and quality
Yes

Improve critical discussion ? **No**

Improve English? **No**

Needs comparative evaluation? **No**

Needs more experimental results?
No

Originality: Newness of the ideas expressed
4

Overall rating **5**

Paper formatting needs adjustment?
No

Presentation: Structure/Length/English
5

References are up-to-date and appropriate?
Yes

Relevance: Paper fits one or more of the topic areas? **4**

Significance: Is the problem worth the given attention? **4**

Technical Quality: Theoretical soundness/methodology **5**

Comment: The paper provides a good discussion and certainly deals with an interesting research topic, and as such, it deserves attention.

Towards Personalized Drug Delivery - Preparation of an Encapsulated Multi-compartment System

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ABSTRACT. Although single artificial vesicles are successfully used as delivery vehicles of pharmaceuticals, unilamellarity and restriction to one vessel result in premature content release in physiological environments as well as problems in simultaneous entrapment of a given set of (pharmaceutical) components. Multilamellarity and assemblies of distinct populations of vesicles are proposed to solve these problems. In this study, we provide a novel encapsulation protocol to fabricate multilamellar vesicles and we report on the DNA-mediated self-assembly of more than two distinct populations of vesicles. We discuss how these results might be used in personalized healthcare based on custom-tailored encapsulated multicompartment vesicular drug delivery systems.

KEYWORDS. personalized healthcare, drug delivery, encapsulation, compartmentalization, programmability, vesicle, liposome

INTRODUCTION

Both biological and artificial vesicles feature an aqueous compartment partitioned from an aqueous surrounding by a lipid membrane that is nearly impermeable to hydrophilic substances. This membrane organizes processes by compartmentalizing them. This compartmentalization enables segregation of specific chemical reactions for the purpose of increased controllability, observability, stability, and biochemical efficiency by restricted dissemination and efficient storage of reactants, and/or reaction products. Hence, vesicle-based Living Technology [1] has gained importance in analytics [2-10], synthetics [11-19], and biomedicine [20-33]. Vesicles featuring biocompatibility, biodegradability, low toxicity, and structural variability are successfully utilized as therapeutic agents for the delivery of antibacterial, antiviral, and anticancer drugs, as well as of hormones, enzymes, and nucleotides [34-36].

The use of single unilamellar vesicles prevails in contemporary therapeutic systems. However premature content release in physiological environments limits their reliability [37]. Extending the circulation time of vesicles that results in passive accumulation at tumors or inflammation sites due to the enhanced permeability and retention (EPR) effect [22] is implemented at the molecular level via monomer design [38] or at the mesoscopic level via encapsulation. The bilayer-within-bilayer structure of encapsulated vesicles not only prevents premature degradation and content release [39] but also offers a division of distinct membrane functions (biocompatibility, cargo release, targeting, and protection) among several membranes of distinct compositions and dimensions.

The applicability of single vesicles is further limited by the need for simultaneous entrapment of a given set of (pharmaceutical) components in one single compartment, which is "not an easy matter" [40, p. 14660]. Multicompartment systems of assembled vesicles can overcome this limitation by conciliating smaller subsets of components entrapped in different compartments.

A combination of encapsulation and (self-

)assembled vesicles may provide stable vehicles for multicomponent or multifunctional drug delivery. Zasadzinski *et al.* [30, 39, 41] established a protocol to encapsulate a multicompartment system of tethered vesicles. Both tethering and encapsulation of these vesosomes are based on the molecular recognition process of the biotin-streptavidin complex. In nature, a multivalent "Velcro-like" system allows for selective tethering of a multitude of different cell types. Cell adhesion molecules (CAMs) that provide this selectivity are emulated in the artificial system by several different linking mechanisms [42-51] (for the latest developments in biomimetic supramolecular chemistry see [52]). The multivalent, selective, and sequence-dependent linkage of nucleic acids has considerable potential since it mimics CAMs and offers programmability to the self-assembly process. Hence, single stranded DNA (ssDNA) is used either to induce assembly of hard sphere [53-56] or vesicular [2, 57-59] colloids, to induce programmable fusion of vesicles [2, 59], or to spontaneously and specifically link vesicles to surface supported membranes [2, 57, 60-64]. However, a linkage of more than two populations of vesicles was not implemented. This lack was remedied only recently by the implementation of a DNA-mediated self-assembly of three distinct populations of vesicles reported here and analyzed in depth elsewhere [65].

In this study, we present both a programmable DNA-mediated linkage of three distinct vesicle populations and a novel encapsulation mechanism. Based on the results of this study, we formulate a scenario how encapsulated multicompartment systems might be used to realize custom-tailored vesicular drug delivery systems.

MATERIALS AND METHODS

Technical modifications of the vesicle formation protocol reported by Pautot *et al.* [66] were: (i) the introduction of 96-well microtiter plates U96 to increase procedural manageability in laboratory experimentation and (ii) a density difference between *inter*- and *intra*vesicular solution induced by isomolar solutions of monosaccharids (glucose: *inter*) and disaccharids (sucrose: *intra*). For a description of the modified vesicle protocol see figure 1.A-D. For the membrane composition of the vesicles used in the encapsulation and the self-assembly experiments see table 1. All phospholipids were dissolved in mineral oil.

For details of the encapsulation procedure of untethered vesicles see figure 1.E-H. Encapsulated vesicles exhibited quick random motion within the boundaries of the surrounding vesicle.

In the compartmentalization experiments, three distinct vesicle populations were prepared that exposed binary combinations of six ssDNA populations on their surface (1st population: α, β; 2nd: α′, γ; 3rd: β′, γ′; for the sequence of biotinylated ssDNA strands see figure 2.E). The DNA strands were biotin-labeled and anchored to biotinylated vesicular membrane via streptavidin as a cross-linking agent. For a detailed protocol of the surface modification and the self-assembly procedure see figure 2.A-D. The sequences of the ssDNA were produced by a genetic algorithm and optimized for minimal DNA-DNA-hybridization among the three pairs.

Light and confocal laser scanning microscopy was performed using an inverted Leica DMR IRE2 SP2 confocal laser scanning microscope.

Results

The geometry of the microplate wells (U shaped-

Table 1: Membrane composition of vesicles used in experimentation.

Encapsulation	
100%	PC(16:0/18:1(Δ9-Cis)) = 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine
Self-Assembly	
99%	PC(16:0/18:1(Δ9-Cis)) = 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine
0.75%	methyl-PEG2000-PE(18:0/18:0) = 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy (Polyethylene glycol)-2000]
0.25%	biotin-PEG2000-PE(18:0/18:0) = 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl (Polyethylene Glycol) 2000]

bottom) and the density difference between the *inter-* and *intra*vesicular solution induced vesicle pelletization at the centre of the well. The size distribution of vesicles produced in the first round of vesicle formation (Fig. 1.A-D) was shifted to the left when compared to vesicles produced in the second round (Fig. 1.E-H). The two vesicle formation protocols technically differed only in the presence (Fig. 1.B) or absence (Fig. 1.F) of the sonication of the water-in-oil emulsion. To indicate independence of the tethering and encapsulation process, vesicles to be encapsulated were not tethered. Tethered assemblies were encapsulated without any modification of the encapsulation procedure (results not shown). As seen in figures 1.K and 1.L the ratio of vesicles internally compartmentalized to vesicles uncompartmentalized was high. Most of the vesicles produced in the first round were found to be enclosed – encapsulation efficiency was high.

When vesicles that exposed complementary ssDNA came into contact, hybridization of single DNA strands resulted in double stranded DNA. Linkers accumulated in the contact area of the two vesicles formed an adhesion plaque (for a schematic representation see figures 2.D/G; for colour-coded micrographs of real-world results see figures 4.D.1

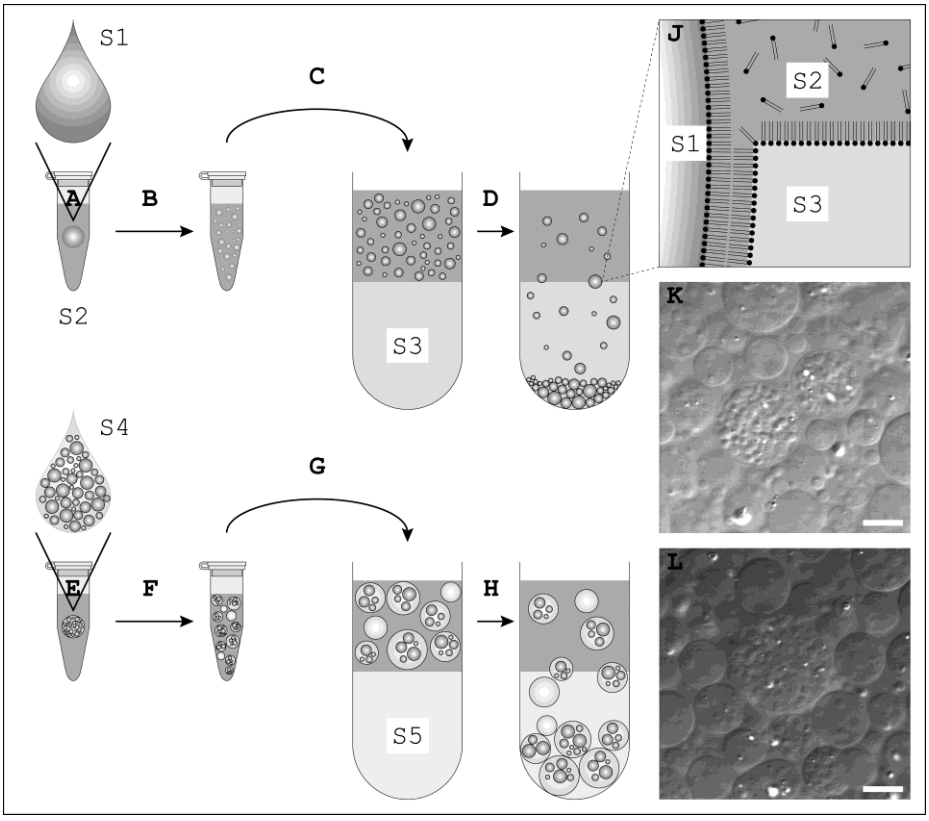

Fig. 1. Schematic representation of the vesicle formation/encapsulation procedure and micrographs of internally compartmentalized vesicles. (A) A water droplet (solution 1, S1) is added to a phospholipid suspension (S2). (B) A water-in-oil emulsion is produced by mechanical agitation and sonication. (C) The emulsion is placed over an aqueous solution (S3). (D) Induced by centrifugation, the droplets pass the oil/water interface. Due to the density difference of the *inter-* and *intra*vesicular fluid and the geometry of the formation chamber, vesicles pelletize in the centre of the well and become easily accessible for pipetting. (E-H) Internally compartmentalized vesicles are prepared by reapplying steps (A-D) using a droplet of the aqueous solution that hosts the vesicles (S4) and an aqueous solution less dense than (S3). (J) Detail of the molecular mechanisms at the water-oil interfaces. Amphiphilic phospholipids, dissolved in mineral oil, stabilize the interfaces by forming monolayers. Two monolayers form a bilayer when a water droplet passes the interface. (K, L) Differential interference contrast micrographs of internally compartmentalized vesicles. Scale bar represents 10μm.

and 5 of [65], figure 5 of [67], and figure 3 of [68]). Adhesion plaques were found exclusively, when DNA strands were complementary and inorganic ions were present (see [65]). No transfer of linkers between the membranes of different vesicles was observed (data not shown).

Discussion

Multicomponent or multifunctional custom-tailored vesicular drug delivery systems have to fulfil several requirements: (i) the actual drug containing system should be encapsulated to prevent premature degradation and content release, (ii) the drug containing system should consist of more than two distinct compartments, and (iii) the proper composition of the drug containing system should be controlled.

Encapsulation

The *in vitro* vesicle formation procedure [12, 66, 69] enables independent tailoring of chemical material properties of the *inter-* and *intra*vesicular fluid as well as of the inner and outer membrane leaflet composition. To our knowledge, the entrapment efficiency of this vesicle formation procedure has not, thus far, been analyzed. However one may speculate that its entrapment efficiency is superior to vesicle formation procedures currently used (for an overview of the current vesicle formation procedures see [10]). The potential of an asymmetric leaf-

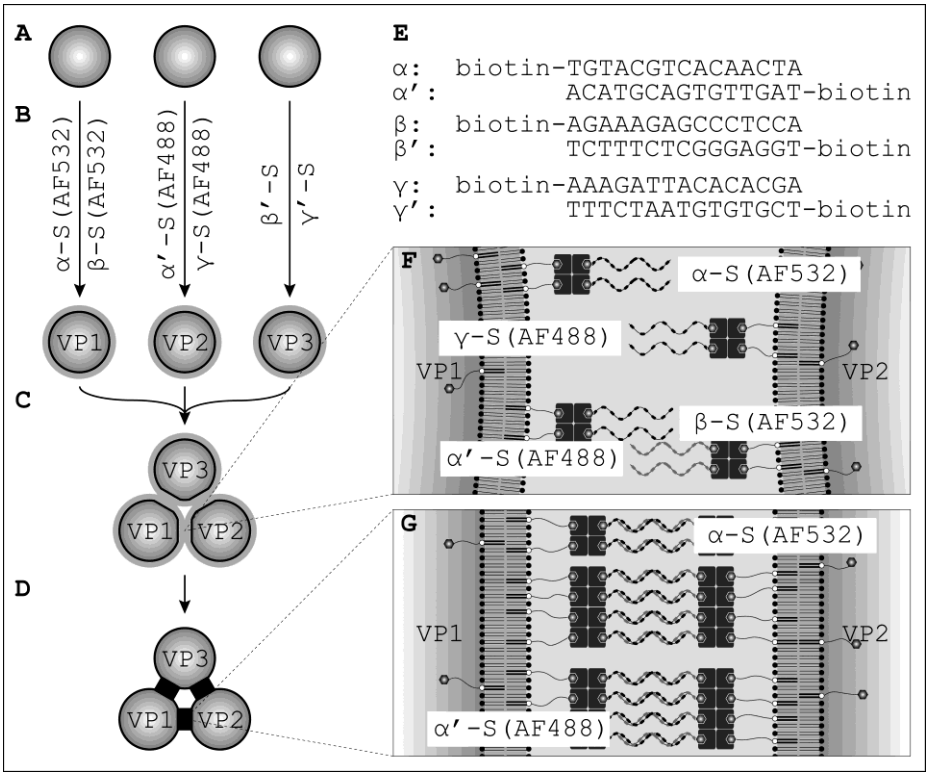

Fig. 2. Schematic representation of the self-assembly process. (A) For the vesicle formation procedure see figure 1. Change: Vesicle membranes incorporate biotinylated phospholipids. (B) Vesicle populations (VP) are labelled (grey ring) by incubation with distinct combinations of biotinylated single stranded DNA (b-ssDNA) that differ in sequence and streptavidin (S) that differ in fluorescence labeling (Alexa Fluor 488 / 532 conjugate (AF488 / AF532) or unlabeled). (C) VPs are merged after excess b-ssDNA/S-solutions are removed. VPs become loosely linked in contact. (D) Linker accumulation at the contact sites (solid black) strengthens the linkage. (E) Sequence of complementary biotinylated DNA single strands used in the self-assembly experiments; only bases G/C and A/T pair. (F) Molecular detail of vesicle labeling. B-ssDNA is linked to biotinylated phospholipids through streptavidin as connector. (G) Sequence specific hybridization of ssDNA results in double stranded DNA (dsDNA) and links VPs. Due to their lateral mobility, linkers accumulate at the contact site. The lateral distribution of linkers in the outer leaflet becomes inhomogeneous (sequence dependent).

let composition was exemplified by the production of phospholipid and polymer hybrids combining biocompatibility and mechanical endurance in single vesicles [66]. We increased procedural manageability of the formation procedure by introducing microtiter plates and vesicle pelletization (due to density differences in the *inter-* and *intra*vesicular fluid). By introducing sonication of the water-in-oil emulsion, we could shift the size distribution of the vesicles formed. By refeeding the vesicle containing solution, we established a novel method to produce multivesicular assemblies. The protocol provides encapsulation of either tethered or untethered vesicular assemblies. The interdependence of tethering and encapsulation, faced in vesosome formation, is therefore resolved.

Compartmentalization

Single stranded DNA provides programmability, specificity, and high degrees of complexity [70]. Streptavidin offers the strongest noncovalent biological interaction known [71], an extensive range

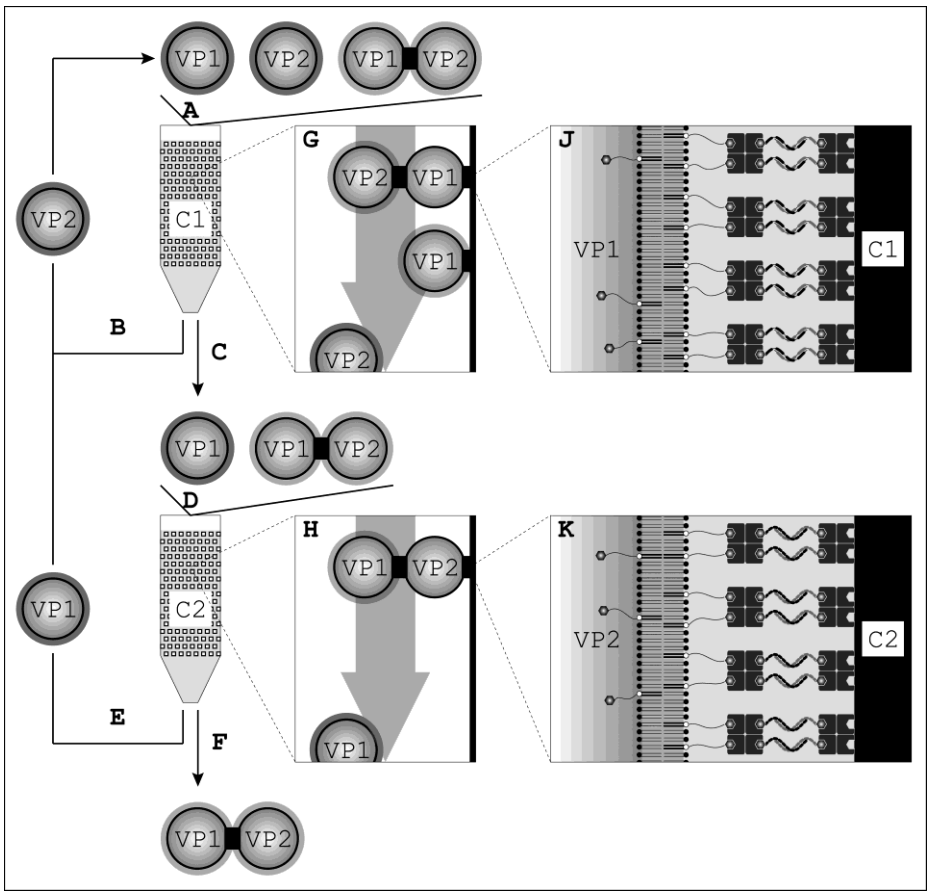

Fig. 3. Schematic representation of the vesicle formation/encapsulation procedure and micrographs of internally compartmentalized vesicles. (A) A water droplet (solution 1, S1) is added to a phospholipid suspension (S2). (B) A water-in-oil emulsion is produced by mechanical agitation and sonication. (C) The emulsion is placed over an aqueous solution (S3). (D) Induced by centrifugation, the droplets pass the oil/water interface. Due to the density difference of the *inter-* and *intra*vesicular fluid and the geometry of the formation chamber, vesicles pelletize in the centre of the well and become easily accessible for pipetting. (E-H) Internally compartmentalized vesicles are prepared by reapplying steps (A-D) using a droplet of the aqueous solution that hosts the vesicles (S4) and an aqueous solution less dense than (S3). (J) Detail of the molecular mechanisms at the water-oil interfaces. Amphiphilic phospholipids, dissolved in mineral oil, stabilize the interfaces by forming monolayers. Two monolayers form a bilayer when a water droplet passes the interface. (K, L) Differential interference contrast micrographs of internally compartmentalized vesicles. Scale bar represents 10μm.

of possible vesicle modifications, component modularity, and availability off the shelf. Phospholipid-grafted biotinylated PEG tethers feature lateral mobility [72], high detachment resistance [73], and no intermembrane transfer of linkers. The combination of phospholipid-grafted biotinylated PEG tethers and streptavidin expedites the production of vesicles avoids problems encountered in other approaches using cholesterol-tagged DNA to specifically link different vesicle populations by the hybridization of membrane-anchored DNA [2, 57, 60]: (i) Because the processes of vesicle formation and vesicle modification are not separated (the cholesterol-tagged ssDNA has to be present during vesicle formation), the formation procedure has to be adjusted anew for each change in the vesicle modification. The procedural manageability in laboratory experimentation is therefore reduced. (ii) As previously reported, the cholesterol anchors of the cholesterol-tagged ssDNA spontaneously leave the lipid bilayer and incorporate randomly into (other) lipid bilayers [57]. Thus, the specificity of the linking system is lost over time.

We have presented a DNA-mediated tethering of three distinct vesicle populations, where the linkage of more than two distinct vesicle populations is reported for the first time. Our findings appear to have solved current restrictions, where donor-acceptor mechanisms are binary. The DNA-mediated linkage mechanism, reported here, offers programmability of composition of multicompartment systems. Thus, custom-tailored vesicular drug delivery systems seem feasible.

Composition Control

By loading the vesicular membranes of tethered assemblies by ligand groups not used in the aggregation process, a column chromatographic purification procedure of aggregates may be realized. The ligand groups would be used to purify aggregates from single vesicles (for details see figure 3.A-F). The scenario represents the minimal situation of tethered assemblies of two vesicle populations and two columns in series. If the tethered assemblies consist of three different vesicle populations bearing three different ligand groups not used in the aggregation process, purification of aggregates of proper composition from both single vesicles and incomplete aggregates may be possible.

By a downstream fluorescence activated cell sorting (FACS; for a review of techniques used in cell separation see Pappas and Wang [74]) internally compartmentalized vesicles may be purified from vesicles not compartmentalized properly. By introducing an intermediate separation process, a refeeding (Fig.3.B/E) of single vesicles and incomplete assemblies into the self-assembly process may be realized before they become encapsulated. This may increase encapsulation efficiency and therefore may economize the production of custom-tailored vesicular drug delivery systems.

Encapsulation provides an extended circulation time resulting in accumulation at tumors or inflammation sites due the EPR effect, without the need of specific targeting. On the other hand, multiple compartments offer segregation of multicomponent pharmaceuticals that might be released only when and where they are needed. Permeability control might be realized either by exploitation of stimuli inherent to target site (pH, redox potential, temperature) or externally induced (temperature, magnetic field, ultrasound). For a recent review on stimuli-sensitive pharmaceutical nanocarriers see Torchilin [38].

Conclusion

Encapsulated multicompartment systems may provide stable vehicles for a multicomponent or multifunctional personalized drug delivery. In this work, we established a novel encapsulation technique and provide evidence for a stable DNA-mediated linkage of more than two vesicle populations. We discussed how these techniques may personalize the individual healthcare by providing custom-tailored vesicular drug delivery systems.

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CHAPTER 8

FUNCTIONALITY: TO GO BACK TO REALITY IN EMBRYOGENIC EVOLUTION

Publication Profile

Title:	Multivesicular Assemblies as Real-world Testbeds for Embryogenic Evolutionary Systems
Year:	2009
Authors:	Hadorn M, Eggenberger Hotz P
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Publication Type:	Book Chapter
Publisher:	Springer-Verlag Berlin Heidelberg
Book Title:	Artificial Life: Borrowing from Biology
Book Series:	Lecture Notes in Artificial Intelligence
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ISSN:	0302-9743
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Maik Hadorn Contributions:	Conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared the figures and tables.

Reviewer Comments

---------- review 1 ----------

OVERALL RATING:	3 (strong accept)
REVIEWER'S CONFIDENCE:	2 (medium)
originality:	5 (excellent)
correctness:	5 (excellent)
English:	5 (excellent)
relevance:	5 (excellent)

This paper describes methods to build a system which could be used to examine embryogenic evolutionary systems in vitro. Two methods, one with latex beads and one with vesicles are described: both succesfully self-assemble groups of elements based on complementary DNA binding. The work appears interesting and significant, but unfortunately the organisation of material makes it difficult to read.

The beginning of the abstract seems to promise that the paper will "check principles that emerged in simulation", yet this is not presented in this paper, only a first step towards possibly doing so is described. Wording here should be revised to avoid such confusions.

Figures are well done, but there appears to be a large amount of information in figure legends which are not present elsewhere in the paper, making it difficult to know in what order to read material. And there are also features in figure legends that should be pointed out elsewhere -- for example, E.1 and E.2 of Fig 1 appear to show how different environments are produced inside and outside vesicles,

but this is not referred to explicitly, although it seems it should be in results. Also, the staining materials (I presume of the vesicile interior) should be mentioned in C, where the staining is first described.

The long introduction intersperses background material with plans for what will be done in this paper with some actual methods of what is done. This should be organised differently, such that background proceeds the plans for this paper, and the methodology (e.g., Fig 1) is described in the methods section. And does mono- versus bi-dispersed simply mean one size versus two sizes? Perhaps this could be said simpler.

---------- review 2 ----------

OVERALL RATING:	1 (weak accept)
REVIEWER'S CONFIDENCE:	2 (medium)
originality:	4 (good)
correctness:	3 (fair)
English:	4 (good)
relevance:	4 (good)

This paper presents some results in chemical self-assembly of vesicles. It is interesting to see some real chemical experiments. However, it isn't clear how this system is evolutionary in any way. I found the diagrams difficult to follow and they seem to consist of repeated elements so don't illuminate much. The captions are too long. Many aspects of the paper are unclear, especially if your audience is not familiar with the chemical systems being used. My advice would be to restructure section 3 in particular - using sub headings to make clear what the results and significance are. At the moment it reads too much like a laboratory description of what you've done.

It is not clear to me how the ssDNA can be "programmed" and exactly how this mediates the self assembly. For example the paper says "Due to the programmable DNA-mediated self-assembly of vesicles (Fig. 3) of designer-specified content (Fig. 1), the emulation of cell differentiation1 becomes feasible. Although vesicles are not able to divide or grow they can be prepared with specific content and then positioned specifically in space. This allows to mimic the end result of a cell differentiation process." -- so that's not really cell differentiation, you're just placing bubbles in specific positions? It seems a big stretch to call this cell differentiation.

---------- review 3 ----------

OVERALL RATING:	2 (accept)
REVIEWER'S CONFIDENCE:	1 (low)
originality:	3 (fair)
correctness:	4 (good)
English:	3 (fair)
relevance:	5 (excellent)

This article discusses using vesicles to create networks of programmable cell-like entities. I am not familiar enough with the detailed chemical/physical systems described to evaluate these aspects, but the general idea and methodology seem sound and highly worthwhile. The novel contribution of this particular article is unclear as the general proposition of using vesicles has been long-standing (many of the references cited are already doing this). The writing could be improved in places.

Multivesicular Assemblies as Real-world Testbeds for Embryogenic Evolutionary Systems

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Abstract. Embryogenic evolution emulates *in silico* cell-like entities to get more powerful methods for complex evolutionary tasks. As simulations have to abstract from the biological model, implicit information hidden in its physics is lost. Here, we propose to use cell-like entities as a real-world *in vitro* testbed. In analogy to evolutionary robotics, where solutions evolved in simulations may be tested in real-world on macroscale, the proposed vesicular testbed would do the same for the embryogenic evolutionary tasks on mesoscale. As a first step towards a vesicular testbed emulating growth, cell division, and cell differentiation, we present a modified vesicle production method, providing custom-tailored chemical cargo, and present a novel self-assembly procedure to provide vesicle aggregates of programmable composition.

Keywords. Embryogenic evolution, real-world testbed, vesicles, hard sphere colloids, DNA, programmability, self-assembly

INTRODUCTION

As evolvability is one of the central questions in artificial life (ALife), artificial evolution (AE) is one of its main aspects. AE is widely used in optimization problems [1]. Evolutionary algorithms (EAs) participating in an AE base on ideas from our current understanding of biological evolution and use mechanisms like reproduction, mutation, recombination, and selection. Since direct encoding schemes where the phenotype of an individual is directly encoded in its genome no longer work for complex evolutionary tasks [2-4], an increasing number of researchers started to mimic biological developmental processes in artificial systems. The genotype-phenotype-mapping becomes indirect by interposing developmental processes. To mimic the natural model in more detail, embryogenic evolution (EE) [5], also called computational evolution [2], simulates cell-like entities that develop into an organism, a neural network etc. Thereby the developmental processes crucially depend on the interactions between the entities typically relying on chemical signaling molecules, membrane receptors, or physical interactions between neighboring cells. EE systems are therefore testbeds to study information guided processes that create higher-order assemblies of cell-like entities, whose overall function emerges from the interaction of their entities.

Banzhaf *et al.* [2] stressed that AE should incorporate physics either in simulation or in real-world experimentation instead of trying to evolve problem solutions in a symbolic way. Although Miller and Downing [6] argued that non-conventional hardware, rather than computer chips, may be more suitable for computer controlled evolution, no real-world experimentation are realized in AE so far. If real biological cells are used to realize a real-world testbed, two arguments have to be considered: Their structure is complex and they consist of a myriad of interacting molecules. In contrast, vesicles are chemically well-defined and therefore easier to analyze and understand. Like real biological cells, vesicles feature an aqueous compartment partitioned off the surrounding by an impermeable lipid

membrane. Like cellular membranes, vesicular membranes consist of amphiphilic phospholipids that link a hydrophilic head and a lipophilic tail (Figure 1.A). Suspended phospholipids can self-assemble to form closed, self-sealing solvent-filled vesicles that are bounded by a two-layered sheet (a bilayer) of 6 nm in width, with all of their tails pointing toward the center of the bilayer (Figure 1.C.1). This molecular arrangement excludes water from the center of the sheet and thereby eliminates unfavorable contacts between water and the lipophilic (= hydrophobic) tails. The lipid bilayer provides inherent self-repair characteristics due to lateral mobility of its phospholipids [7]. As vesicles vary in size from about 50 nm to 100 μm, mechanisms of interest such as self-assembly or information processing can be tested over a wide range of scale. This scale range, called mesoscale, is not yet very well understood scientifically. Many of the biological concepts fall in this mesoscale range; the proposed testbed may therefore be of special interest in ALife. Their minimality and self-assembling and self-sealing properties make vesicles an excellent candidate for a testbed in which concepts of EEs can be set up and tested in the real world. Elaborated *in vitro* vesicle formation protocols [8-10] provide independent composition control of the *inter-* and *intra*vesicular fluid as well as of the inner and outer bilayer leaflet. Asymmetry in the inner and outer bilayer leaflet was realized by the production of phospholipid and polymer vesicles combining biocompatibility and mechanical endurance [9]. As a result of the analogy to natural systems and the compositional simplicity, artificial vesicles are the

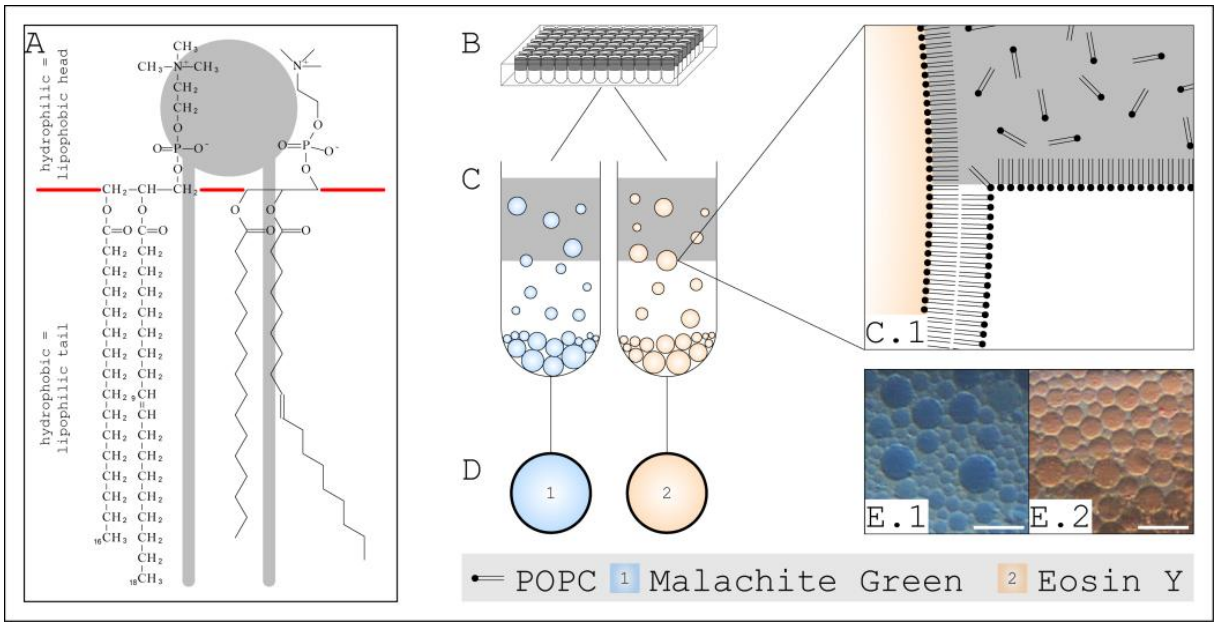

Fig. 1. Production of vesicles differing in their content. (*A*) The chemical structure of the phospholipid POPC (= PC(16:0/18:1(Δ9-Cis)). PC(16:0/18:1(Δ9-Cis) is represented as a structural formula (front, left), as a skeletal formula (front, right), and as a schematic representation (back). The schematic representation is used throughout this publication. For a discussion of the relevance of the amphiphilic character of phospholipids in the formation of biological and artificial membranes see text. (*B*) Microwell plate in which the vesicles are produced. (*C*) The sample is composed of two parts: distinctly stained water droplets (Malachite Green and Eosin Y) in the oil phase and the bottom aqueous phase, which finally hosts the vesicles. (*C.1*) Due to their amphiphilic character, phospholipids, dissolved in mineral oil, stabilize water-oil interfaces by forming monolayers. Two monolayers form a bilayer when a water droplet, induced by centrifugation, passes the interface. Due to both the density difference of the *inter-* and *intra*vesicular fluid and the geometry of the microplate bottom, vesicles pelletize in the centre of the well. (*D*) The two vesicle populations differ only in intravesicular staining. (*E.1*, *E.2*) Light microscopic visualization of the two distinct vesicle populations stained by Malachite Green (*E.1*) and Eosin Y (*E.2*) that emerged in real-world experimentation. Staining persisted for days. Scale bars represent 100μm.

most studied systems among biomimetic structures [11] providing bottom-up procedures in the analysis of biological processes. Starting with distinct vesicle populations differing in content, size, and/or membrane composition, self-assembly of vesicle aggregates providing programmability of composition and structure may emulate growth, cell division, and cell differentiation. The potential of a programmable self-assembly of superstructures with high degrees of complexity [12] has attracted significant attention to nanotechnological applications in the last decade. The high specificity of binding between complementary sequences and the digital nature of DNA base coding enable the programmable assembly of colloidal aggregates. So far, cross-linkage based on DNA hybridization was proposed to induce self-assembly of complementary monohomophilic vesicles [13, 14] or hard sphere colloids [15-18], to induce programmable fusion of vesicles [14, 19], or to specifically link vesicles to surface supported membranes [14, 20-22].

Here, we present a new protocol for *in vitro* vesicle formation and membrane modification that increases the versatility of the underlying vesicle formation method by introducing microtiter plates and vesicle pelletization. Asymmetry in the *inter-* and *intra*vesicular fluid was realized by vesicle staining. We contrast assemblies of hard sphere colloids and multivesicular aggregates to research the influence of material properties on the creation of higher-order assemblies of cell-like entities. We discuss how an asymmetry in intravesicular fluids in combination with vesicular assemblies of preprogrammed structure and composition may emulate cell differentiation and may be used as a real-world testbed for EE systems on mesoscale.

MATERIAL AND METHODS

We performed self-assembly experiments of colloidal particles and vesicles in real-world. The self-assembly process was based on the hybridization of single-stranded DNA (ssDNA) with which the surfaces were doped (Figures 2, 3). By introducing a surface doping of distinct populations of ssDNA, as realized in the self-assembly of hard sphere colloids [23, 24], n-arity may be provided to the assembly process.

Technical modifications of the vesicle formation protocol reported by Pautot *et al.* [9] were: (i) the introduction of 96-well microtiter plates U96 to increase procedural manageability in laboratory experimentation and (ii) a density difference between *inter-* and *intra*vesicular solution to induce vesicle pelletization. Solutions of the vesicle lumen and the surrounding medium were equal in osmolarity but differed in the size of dissolved saccharides (lumen: disaccharides, environment: monosaccharides) providing density differences between the lumen and the environment. For a description of the modified vesicle protocol see Figure 1. Vesicles were either made of 100 percent PC(16:0/18:1(Δ9-Cis)) (staining experiments, Figure 1) or 99 percent PC(16:0/18:1(Δ9-Cis)) and one percent biotin-PEG2000-PE(18:0/18:0) (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol) 2000]) (self-assembly experiments, Figure 3). Doping of the vesicular surface was realized by anchoring biotinylated ssDNA to biotinylated phospholipids via streptavidin as a cross-linking agent. The mean particle size of the latex beads constituting the hard sphere colloid was 0.25 or 1.0 μm (Figure 2). Their surface was streptavidin labeled off the shelf. The sequences of the DNA single strands were the same for the colloidal and vesicular self-assembly experiments. For a detailed protocol of the surface doping procedure see Figures 2 and 3. The sequence of complementary biotinylated ssDNA strands (α: biotin-TGTACGTCACAACTA-3', α': biotin-TAGTTGTGACGTACA-3') were produced by a genetic algorithm. Light and confocal laser scanning microscopy was performed using a Wild M40 inverted microscope and an inverted Leica DMR IRE2 SP2 confocal laser scanning microscope.

RESULTS AND DISCUSSION

Asymmetry in the Inter- and Intravesicular Fluid

Vesicles were found to sediment and hence to be easily available for inverse microscopy. Asymmetry of *inter-* and *intra*vesicular fluid (vesicle staining, Figure 1.E) persisted for days (data of long-term observation not shown). Although vesicles are not able to differentiate, they can be prepared with specific content and then positioned specifically in

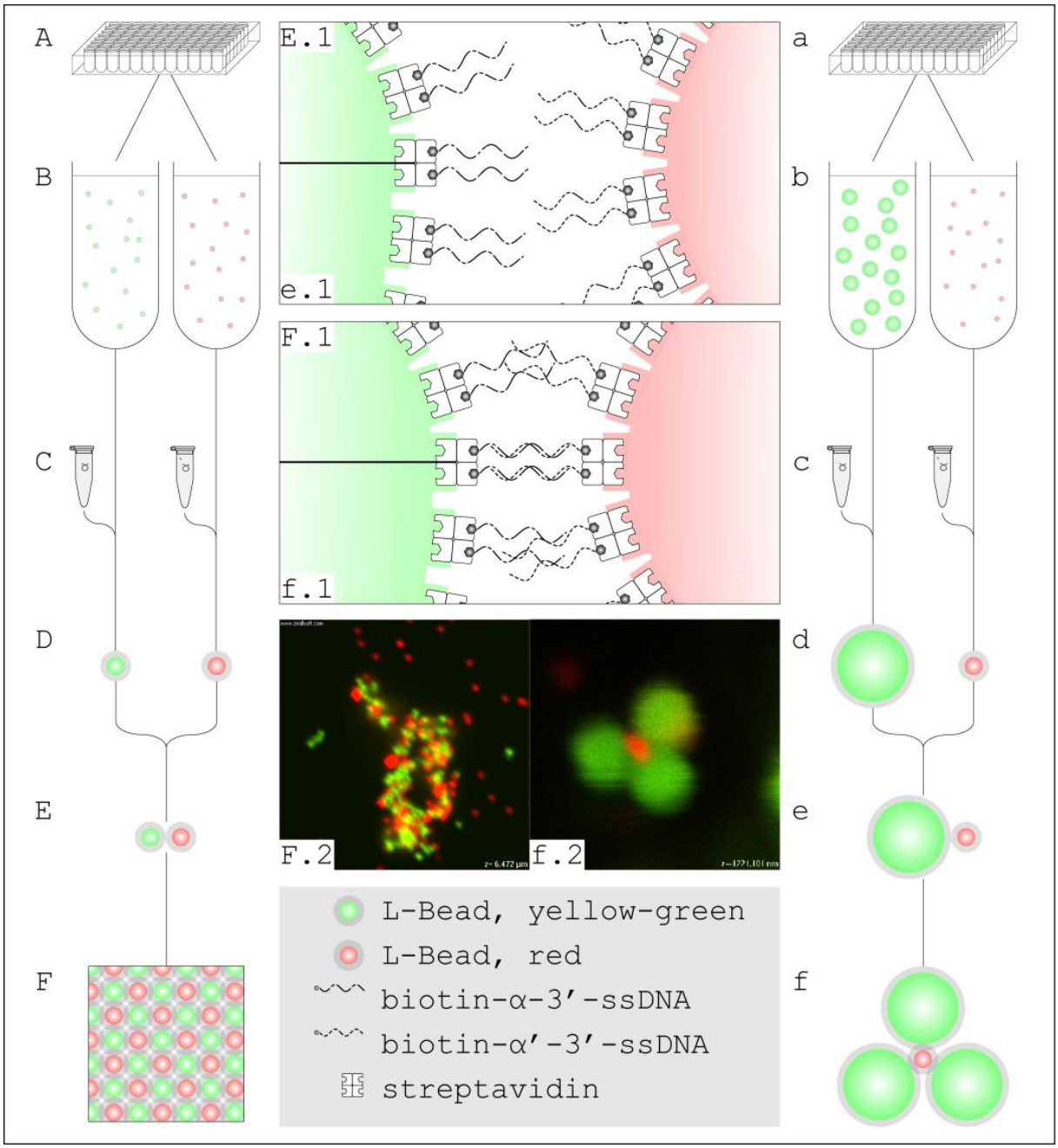

Fig. 2. Schematic representation of the surface doping procedure and the self-assembly process of mono- and bidispersed spherical colloids. (*A*,*a*) Surface doping and self-assembly are realized in 96-well microtiter plates, providing parallel modification of up to 96 distinct bead populations. (*B*,*b*) The two populations of latex beads differ in fluorescent labeling (yellow-green, red). (*C*,*c*) The surfaces of the latex beads are homogenously labeled with streptavidin. The two populations of beads are doped with single stranded DNA (ssDNA-α and ssDNA-α′) of complementary sequence (α, α′). (*D*,*d*) ssDNA covalently bound to biotin is non-covalently linked to the streptavidin labeled surface. (*E*,*e*) The two bead populations become merged (brace). (*E.1*,*e.1*) Beads come into contact. (*F*,*f*) Hybridization of DNA strands results in double stranded DNA and induces the assembly process. The self-assembly of monodispersed bead populations results in non-terminating aggregates (*F*), whereas aggregates are terminating in assemblies of bidispersed bead populations (*f*). (*F.1*,*f.1*) The lateral distribution of linkers is not affected by the assembly process (cp. Figure 3). (*F.2*,*f.2*) Confocal laser scanning microscope visualization of the aggregates that emerged in real-world experimentation.

space. This allows to mimic the final result of a cell differentiation process.

Assemblies of Hard Sphere Colloids and Multivesicular Aggregates

In assemblies of two bead populations of equal concentration and size, large clusters of beads exhibiting regular patterns of red and green emerged (Figure 2.F.2). If the bead populations differed in size and if the concentration of the larger beads exceeded the concentration of the smaller ones by a factor 10, small clusters were observed (Figure 2.f.2).

When vesicles doped with complementary ssDNA came into contact, linkers accumulated in the contact phase forming an adhesion plaque (Figure 3.G). Thus, the lateral distribution of linkers in the outer leaflet becomes inhomogeneous as a result of the self-assembly process (cp. [25]). It is conceivable that an accumulation of linkers may result in self-termination of the assembly process. Thus, in contrast to assemblies of hard sphere colloids the size of the aggregates may be adjusted by variations in surface linker density. Adhesion plaques were found exclusively, if DNA strands were complementary and monovalent ions were present (data of control experiments not shown). No transfer of linkers between the membranes of different vesicles was observed (data not shown). ssDNA provides programmability, specificity, and high degrees of complexity [12]. Streptavidin offers the strongest noncovalent biological interaction known [26], an extensive range of possible vesicle modifications, component modularity, and availability off the shelf. Phospholipid-grafted biotinylated PEG tethers feature lateral mobility [7], high detachment resistance [27], and no intermembrane transfer of linkers [13, 20]. The combination of phospholipid-grafted biotinylated PEG tethers and streptavidin allows fast production of vesicles differently doped and avoids problems encountered in other approaches using cholesterol-tagged DNA to specifically link different vesicle populations by the hybridization of membrane-anchored DNA [14]: (i) Because the processes of vesicle formation and vesicle modification are not separated (the cholesterol-tagged ssDNA have to be present during vesicle formation), the formation procedure has to be adjusted anew for each change in the vesicle modification. The procedural manageability in laboratory experimentation is reduced therefore. (ii) As discussed by Beales and Vanderlick [13] the cholesterol anchors of the cholesterol-tagged ssDNA spontaneously leave the lipid bilayer and incorporate randomly into (other) lipid bilayers. Thus, in contrast to our linking mechanism specificity of linking is lost when using cholesterol-tagged ssDNA.

How to Implement a Real-world Testbed for Embryogenic Evolutionary Systems on Mesoscale

By using vesicles as entities of a real-world testbed for embryogenic evolution, some aspects of the developmental processes may be investigated and compared at the right scale (nano- and micrometer scale). At this mesoscopic scale, the physics are no longer intuitive. This makes such a testbed a valuable tool to understand the workings of (implicit) physical processes.

Due to the programmable DNA-mediated self-assembly of vesicles of designer-specified content, the emulation of cell differentiation becomes feasible. Although vesicles are not able to divide or grow, they can be prepared with specific content (see section 0), positioned in space (see section 0), and their content can be released on an external trigger [28] and may serve as signaling molecule triggering other processes. Already Noireaux and Libchaber [8] incorporated a transcription-translation cell-free system into vesicles and were able to induce protein synthesis. The exchange of material between the vesicles is provided by inducing pores in the membrane. To induce pores several methods are available such as electroporation [29], phase transitions [30, 31], or protein channels/transporters [32].

We think that such a vesicular system will provide main aspects of intercellular communication and cell differentiation, but in contrast to biological systems, it would be simpler and better defined and therefore easier to be understood.

CONCLUSION

In this work we proposed to use multivesicular assemblies to test principles of EE systems in a real-

world *in vitro* testbed. We developed suitable production methods for vesicles differing in chemical content and doped with different DNA-addresses and presented results of self-assembled multivesicular aggregates, as a prototypical example of information processing in distributed cellular systems. Although we still have a long way to go for self-assembling and working vesicular clusters with programmable and designer tailored properties, we

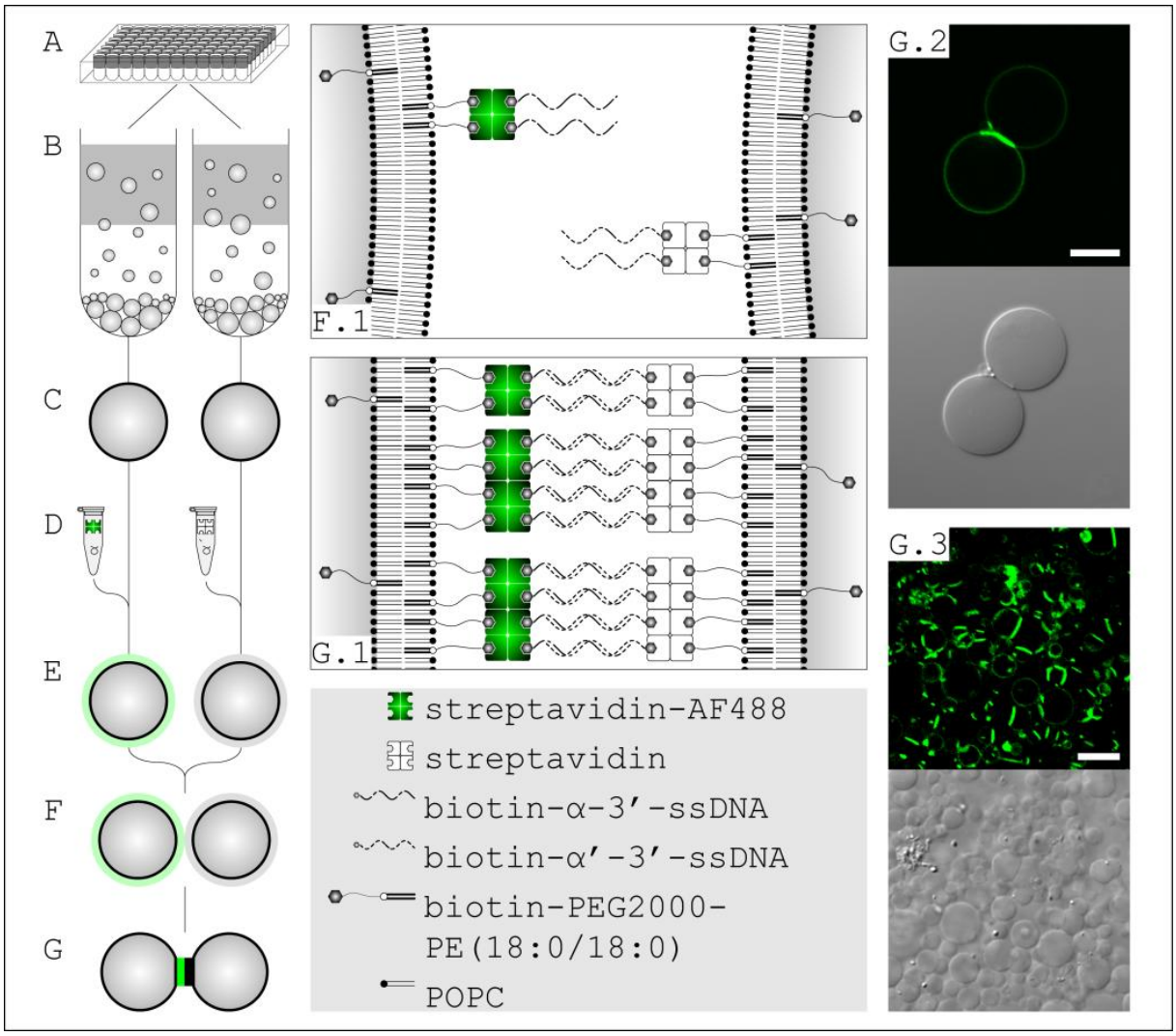

Fig. 3. Schematic representation of the membrane doping procedure, the vesicular self-assembly process and micrographs of adhesion plaques. (*A-C*) Vesicle formation: for details see Figure 1. Vesicles remain on the same microtiter plate during formation and membrane doping. (*D*) Vesicle populations become distinct by membrane doping. The membrane of the vesicles is doped with two complementary populations of single stranded DNA (ssDNA). (*E*) ssDNA covalently bound to biotin is non-covalently linked to phospholipid-grafted biotinylated polyethylene glycol tethers using streptavidin as cross-linking agent (cp. *F.1*). The vesicle populations differ in fluorescence labeling of the streptavidin (Alexa Fluor 488 conjugate (AF488)). (*F*) The vesicle populations become merged (brace). (*F.1*) The lateral distribution of linkers in the lipid membrane is homogeneous. Vesicles come into contact. Hybridization of DNA strands results in double stranded DNA and induces the assembly process. (*G*) Due to their lateral mobility, linkers accumulate in the contact zone forming an adhesion plaque – linker density *inter* adhesion plaques is reduced due to depletion. (*G.1*) Biotinylated phospholipids of the outer leaflet colocalize with the linkers. (*G.2*,*G.3*) Confocal laser scanning microscope (CLSM) and differential interference contrast micrographs of vesicular aggregates that emerged in real-world experimentation. Accumulation and depletion of linkers are clearly visible in the CLSM micrograph. Scale bars represent 10μm.

think that our results illustrate a promising step towards interesting applications such as vesicular nano-robotics, adaptive materials or programmable chemical fabrication tools. Vesicles are scale invariant and it is easy to produce vesicles from 50 nm up to 100 μm. Thus, one may transfer mechanisms investigated on the microscale also to the nanoscale.

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CHAPTER 9

HOW FPAACS RESHAPE THE WAY WE THINK: A NEW VIEW OF COMPUTATION

1 Scientific Significance

LT should create a qualitative jump in man-made systems by exploiting the characteristics of natural systems such as the ability to maintain and repair oneself, to act autonomously, to reproduce, and to evolve adaptively. As technologies increasingly embody such core aspects of living systems, they become increasingly powerful, natural, and sustainable. The results of this thesis, however, may convince people that even non-living systems are qualified as a promising branch of LT with a raison d'être of their own.

Novel experimental protocols that increase procedural manageability, allow for high-throughput analyses of parameters affecting artificial vesicles, and allow vesicles to be internally structured by artificial organelles constitute technical progress.

The concepts and design formulated and tested mark the first step toward FPAACs, which exploit the material and morphological properties of the components to provide programmability, information processing, and functionality. By pointing out the importance of the material and morphological properties, this thesis heavily contributed to change or even to (re)set the course of completed (EES, PACE), current (MATCHIT), and future LT research, while reshaping our notion of computation. As a consequence, the chapter heading pays tribute to Pfeifer's seminal book 'How the Body Shapes the Way We Think – A New View of Intelligence' [1].

1.1 Progress in Experimentation

The new protocol for *in vitro* vesicle fabrication eases procedural manageability. The introduction of microtiter plates enabled the implementation of high-throughput analyses of parameters affecting artificial vesicles (chapters 3, 4, 5, Appendix 1). The independent composition control of the *intra-* and *inter*vesicular fluid enabling vesicle pelletization may complement the more challenging vesicle preparation currently applied [2] and offers a new methodology to prepare internally compartmentalized vesicles (chapter 7).

The new protocol for vesicle self-assembly resulted in the first implementation of a multi-vesicle structure of defined architecture. The linkage process based on the hybridization of biotinylated ssDNA anchored to the vesicular surface through streptavidin and phospholipid-grafted biotinylated PEG tethers was implemented for the first time (chapter 4). The new linkage methodology not only guarantees specificity, but it merges the programmability and high degrees of complexity of DNA hybridization with the strongest non-covalent biological interaction known, an extensive range of possible vesicle modifications, component modularity and availability of streptavidin; moreover, the long and flexible phospholipid-grafted biotinylated PEG tethers provide mobility, high detachment resistance and no intermembrane transfer of linkers. Because vesicles expose biotinylated PEG tethers on their membrane, universal anchor sites are offered. This allows for constancy in the vesicle preparation protocol and for a multitude of different vesicle modifications. As a consequence, there is no need to adapt vesicle fabrication according to different vesicle decorations (chapter 2). By identifying sodium iodide as the monovalent anionic species hindering vesicle stability the least (chapter 3), the long-lasting problems in providing specificity to the self-assembly process are minimized.

1.2 Progress in Concept and Design

According to the '1st International Conference on Morphological Computation' that took place at the European Center of Living Technology (ECLT, Venice, Italy) in 2007, computation in general is defined by (i) programmability of the process, (ii) a clearly identifiable input and output, and (iii) a 'teleological embedding', i.e., the system has to have some kind of desired functionality. To consider this definition, the programmability of matter and morphology, input-process-output, and functionality of the wetware system will be discussed individually and contrasted to ideas either reported elsewhere or discussed in the course of this thesis.

1.2.1 Programmability

LT and synthetic biology share the common objective of designing and constructing new biological functions and systems not found in nature. Waclaw Szybalski coined the term and the concept of 'synthetic biology' in 1974, when writing [3]: *"Let me now comment on the question 'what next'. Up to*

now we are working on the descriptive phase of molecular biology. […] *But the real challenge will start when we enter the synthetic biology phase of research in our field. We will then devise new control elements and add these new modules to the existing genomes or build up wholly new genomes. This would be a field with the unlimited expansion potential and hardly any limitations to building 'new better control circuits' and* […] *finally other 'synthetic' organisms, like a 'new better mouse'.* […] *I am not concerned that we will run out exciting and novel ideas,* […] *in the synthetic biology, in general."* By focusing on the genetic (re)programming of existing organisms, synthetic biology pursues the strategy of synthesizing new or modified substances with the following aims: (i) to test our current understanding of natural living systems, synthetic biology research by following a bottom-up approach in accordance to the synthetic methodology, i.e., a methodology that seeks to understand natural living systems by artificially building or modifying them; (ii) to extend and apply synthetic chemistry to biology, i.e., to include work ranging from the creation of useful new biochemicals to studying the origins of life; and (iii) as an comprehensive scheme, to engineer biological systems that process information, manipulate chemicals, fabricate materials and structures, produce energy, provide food, and maintain and enhance human health and our environment. For a recent review on synthetic biology and its potential applications see [4]. Because synthetic biology mainly refers to genetic (re-)programming of existing organisms, its focus is narrower than LT, which pays attention to choosing materials in a sophisticated way, to program matter in the strict sense by exploiting its inherent properties.

This programmability of matter brings LT closer to ICT. The exploitation of material properties in 'evolvable hardware' became one aspect of modern ICT when reconfigurable hardware (FPGA) became available to ICT in the 1990s [5]. The current state of the art in LT is, therefore, comparable to ICT twenty years ago. Evolvable hardware was debated in ICT for years, but only the invention of FPGAs made it feasible [6].

Banzhaf [7] and Miller and Downing [8] proposed a combination of ICT and LT some years ago, i.e., a combination of real-world experimentation and non-conventional hardware. However, the PACE and EES projects were the first efforts that combined ICT and LT to allow for an ICT-aided programmability of artificial cells. These efforts helped to categorize LICT research, to gain insights into the notion of computation, and to characterize aspects to be addressed in future work. Concerning the physical embodiment of ICT problems in LT, the PACE consortium distinguished the two following types of concepts [9]: (LICT-a) The problem is digital, processed in wetware, and the result is translated back into a digital form; (LICT-b) neither the problem nor the result are digital, but the problem representation (input), information processing, and storage of results (output) are in wetware itself. To represent the problem in wetware and to translate the result back into a digital form, the medium of computation must provide discrete states. Hence, LICT-a is implemented using DNA or other molecules that offer Boolean operations. For an impressive example, see the implementation of a Sierpinski triangle (cf., rule 90) using DNA molecules by Rothemund *et al.* [10]. Like in classical ICT, the analogous effects caused by the physico-chemical properties of the wetware system, i.e., the concentration or temperature dependence and the likelihood of interaction should be eliminated; because the input-process-output steps are digital, LICT-a capitalizes on nature and its laws solely by exploiting a highly parallel manner of computation, but the problems themselves are computable in pure ICT systems. LICT-a offers therapeutic and diagnostic applications and for nanoscale electronic engineering (for a recent review see [11]). However, the constraints previously discussed may cause bottlenecks in future implementation and in versatility.

LICT-b focuses on the generation of a new ICT embedded in LT rather than on the transfer of classical ICT to a new medium as in LICT-a. The analogous effects caused by physico-chemical properties of the wetware system are expressively exploited in LICT-b.

For ICT, Banzhaf *et al.* [7] stressed the incorporation of physics as one of the main points on the research agenda to develop the new field of computational evolution that *"could solve previously unimaginable*

or intractable computational and biological problems". The lack of a field-programmable wetware system forced ICT researchers to map physical reality underlying the process onto a digital representation to allow for exploitation of physico-chemical principles *in silico* [12]. However, despite the numerous insights they provide, the simulations inherently have to abstract from aspects of the real-world. Hence, even well-elaborated simulations and reproducing current knowledge may overlook the sophisticated strategies of nature, because the core features of the model are implicit to the components and their interaction. This reality-gap problem [13] is considered as one of the major challenges to predict real-world behavior when using simulations. Moreover, in ICT, calculating the dynamics of a non-linear system is considerably more difficult than of a linear one.

In wetware, there is no reality-gap problem, because the system is grounded (cf., the symbol-grounding problem, [14]) and the distinction between linear and non-linear systems is not relevant, because both processes just take place following the laws of physics. As pointed out above, research in LICT-b has to deal with myriads of material properties and physical constraints; to provide programmability, LICT-b has to capitalize on concepts like self-organization and emergence exploiting properties, and constraints inherently offered by the materials. Self-termination of the self-assembly process, by exploiting the membrane properties and consequently the programmability of architecture presented in this thesis, properly exemplifies the concepts of programmability of matter that were identified to take center stage in LICT-b.

Hand-made networks of vesicles [15-17] or controlled collision, adhesion, and fusion of vesicles, as proposed in MATCHIT and implemented for nanoliter droplets in microfluidic devices [18], represent impressive examples of technical feasibility, but they are costly either in terms work or control load. This centralized control to specify morphology is required as long as multivalent ligands providing specificity to the self-assembly process are unavailable. The multi-vesicle assemblies of programmable architecture implemented for the first time in this thesis represent crucial progress towards a new age of LICT-b. Like the self-organization of cells of embryonic tissues in nature, mingled vesicles that expose distinct DNA strands on their surface sort out spontaneously without the need of any external or central control.

For an in-depth discussion, the programmability of matter is differentiated into the following three categories: programmability at the molecular scale, programmability at the supramolecular scale, and the programmability of communication.

1.2.1.1 Programmability at the Molecular Scale

In the spontaneous sorting process of vesicles, programmability refers to the sequence of DNA strands. The sequence of the nucleobases adenine (A), thymine (T), guanine (G), and cytosine (C) of a DNA strand defines the sequence of its complementary strand because each type of base on one strand forms a bond with just one type of base on the other strand, with A bonding only to T, and C bonding only to G. However, DNA not only becomes programmable due to its unique feature of sequence specific bonding. The strength of interaction is also programmable by the length of the strands. This direct link of strand length and double strand stability will be exploited (cf., chapter 7) when multi-vesicle assemblies are released from the column without destroying the vesicle-vesicle linkage, even though the linkage to the column and vesicle-vesicle cross-linking are based on the hybridization of DNA single strands.

Alternative to the self-organized self-termination resulting from the supramolecular processes discussed below, two more 'centralized' ideas were discussed but abandoned for this work, namely (i) controlled DNA-induced pairing of vesicles in the microfluidic device, as discussed above, might be followed by the addition of short complementary ssDNA that consume the DNA strands that were not used up in the vesicle-vesicle linkage. Like the depletion of linkers resulting from the linkage-induced accumulation of linkers, this procedure would prevent further growth of self-assembled structures and hence terminate self-assembly. (ii) DNA nanotechnology offers convenient, reliable, and programmable DNA molecular switches or motors that

are based either on a pH-driven strategy [19] or on an electrical actuation [20]. DNA single strands either fold back on themselves, thus becoming inaccessible for pairing, or they are stretched and available for linkage. Vesicle chemistry might be complemented by this technology, thereby offering an external control of the 'stickiness' of vesicles. In contrast to examples where addresses exposed do not change in sequence, MATCHIT will go even further because the addresses themselves will be chemically modifiable and will be dynamic in response to vesicle fusion history.

1.2.1.2 Programmability at the Supramolecular Scale

In addition to the molecular properties, supramolecular features were exploited in this work to provide the programmability of morphology. Even if distinct populations of hard sphere colloids expose distinct multivalent ligands, static anchorage prevents a linkage-induced accumulation of linkers at the adhesion site, as reported for vesicles in great detail (cf., chapters 4, 5, 7, 8); therefore the vesicle self-assembly process is self-terminating whereas the assembly of hard sphere colloids is not. The mobility of the linkers self-terminates the self-assembly process, because the residual membrane becomes deprived of ligands. Supramolecularity refers both to the mobility of ligands and to the formation of adhesion plaques as a result of the interaction of vesicles complementary to the DNA sequence.

The coordination number or stoichiometry of the vesicles in the self-assembly process in relation to the ligand surface density was not quantitatively evaluated in this thesis. However, it is tempting to hypothesize that the absolute number of ligands controls the number of cross-links a vesicle will form by self-organization. To clarify this point, imagine the following situation: Two vesicle populations exposing single strands of DNA with complementary sequence are available. The absolute number of ligands in one population is half compared to the other population. Moreover, we assume that the amount of ligands lasts for the formation of one or two adhesion plaque(s), respectively. Thus, while one vesicle gets depleted of ligands when an adhesion plaque is formed, its partner is still able to form a second adhesion plaque. Like in H_2O, a stoichiometric ratio of vesicles of 2:1 is hence feasible. In 1994, Chiruvolu *et al.* [21] proposed to predetermine the stoichiometry of vesicle structures by loading each vesicle with a finite number of ligand groups. However, to our knowledge, only in this work was a 1:1 as well as a 1:1:1 stoichiometric ratio in vesicle assemblies reported (see figure 5 of chapter 4).

Other instances of programmability at the supramolecular scale are cell or vesicle membranes. Initiated by the theoretical work of Păun [22], who proposed to use several cell-like membranes recurrently placed inside a unique 'skin' membrane, membranes are considered to have great potential for implementing massively concurrent systems in a way that would efficiently solve currently intractable problems. Within the enormous body of research on membrane computation, the vast majority has been dedicated to simulation work. As Păun writes [23], *"*[it] *is important to underline the fact that 'implementing' a membrane system on an existing electronic computer cannot be a real implementation, it is merely a simulation. As long as we do not have genuinely parallel hardware on which the parallelism* […] *of membrane systems could be realized, what we obtain cannot be more than simulations, thus losing the main, good features of membrane systems". "Going back to reality"* was, therefore, recognized as the real challenge, causing a growing demand for genuine hardware that is optimized for artificial chemistries and membrane systems. Shaw *et al.* [24] tackled this challenge by using glass beads and a brass membrane. They showed that the sorting processes across membranes depend on the geometry of the pores of the membrane. The PACE consortium commended this result as an instance of morphological computation [9]. More sophisticated examples of the implementation of membrane computing in hardware using the reconfigurable circuits of FPGAs were reported by Petreska and Teuscher [25]. The obvious use of natural membranes is not addressed so far. Because the new wetware experimental protocol elaborated in this thesis allows to the internal structuring of vesicles by artificial organelles, prerequisites were created such that membrane computing can get wet (again).

Cell differentiation during the developmental

processes of natural organisms results in cell types being spatially arranged and orchestrated. Because the asymmetric cell division known to be important in morphogenesis [26, 27] is not achieved in the replication of vesicles [28, 29], replicating single vesicles cannot be used to mimic these developmental processes. Programmable assemblies of vesicles distinct in membrane composition and/or content may bridge this gap for now.

The control of architecture (Chapter 4), as well as membrane compositions (chapter 8), was accomplished in this work. Concerning the control of content, the incorporation of dyes (Chapter 8), sugars (e.g., Chapter 3), nucleotides (Appendix 1), and organic (Appendix 1) and inorganic (Chapter 3) monovalent salts may emulate the asymmetric spatial distribution of factors affecting development. In artificial systems, asymmetric cell division could thus be replaced by the positioning of vesicles in space and time.

1.2.1.3 Programmability of Communication

In addition to the self-organization of matter, the implementation of spatially and temporally programmable communication was proposed (Chapter 6).

Current protocols implementing the communication of adjacent volumes provide either tube-like channels that are always open like the hand-made networks of vesicles [15-17] or one-shot communication by the irreversible fusion of vesicles [30, 31].

Because the placement of substrates and catalysts, as well as the properties of communication paths, are predefined by the programmable architecture of a Multi-Compartment Communication Network (MCCN), the stepwise routing of educts through the network, the chemical reactions they undergo, and the substances produced are highly controllable and programmable. The MCCNs proposed will benefit from programmability of both the network's architecture and communication pathways. Only adjacent vesicles communicate through the adhesion sites. Thus, the network's architecture defines the communication channels. The opening and closing of the channels are externally controlled by defining the state of temperature. The programmable flow and fusion of the vesicles in microfluidic channels, as proposed for the completed and current efforts of the European Commission (cf., PACE and MATCHIT), may be complemented by a programmable flow of substances from vesicle to vesicle within a MCCN. In addition to the self-organized self-assembly of such MCCNs and the resulting increase in adaptivity, robustness, and versatility compared to the microfluidic technology, miniaturization may accompany these systems. Vesicle membranes constitute a veritable barrier to the passage of most substances. Hence, asymmetries across the membrane are less subject to the balancing effect of diffusion than open systems. By using vesicles, asymmetries in content composition are, therefore, sustainable on smaller length scales and longer time scales than in microfluidic devices.

Hemifusion structures in association with half-membrane spanning transmembrane channels may supplement the communication discussed for the phase transition of membranes in MCCNs. Hemifusion was investigated in the early stages of the thesis (cf., Figs. 1 and 2 in Chapter 3) as an unspecific linking mechanism of vesicles. It represents connections between the outer leaflets of apposed membranes, while the inner leaflets remain distinct. Because a hemifusion connection is often a transient structure that either dissociates or gives rise to a fusion pore (for a recent review on the mechanics of membrane fusion see [32]), this idea was abandoned but assessed as worth pursuing in future work concerning communication. In particular, the asymmetry in the leaflets as reported by Pautot *et al.* [2] may be used to prepare inner leaflets hosting half-membrane spanning transmembrane channels (e.g., Gramicidin A, [33]). At the adhesion sites of hemifused vesicles, the inner leaflets of adjacent vesicles form a new double layer (see 'hemifusion diaphragm' in Fig. 1.a.iv of [32]). Because the half-membrane spanning transmembrane channels have to form dimers that come together end to end across the lipid bilayer to form a permeable transmembrane channel, communication takes place only at the adhesion sites.

1.2.2 Input-Process-Output

In the instances discussed above, programmability refers to the choice of 'proper' materials for which

we already have information regarding how they react under certain conditions, i.e., DNA strands of complementary sequences exposed on different vesicle membranes cross-link merged vesicles on the condition that the concentration of salt ions is above the minimum and temperature is between the freezing of water and the melting temperature of the hybridizing DNA strands; the choice of phospholipids that differ in length and saturation of their hydrocarbon chains, as well as in head group modification (linked to ssDNA: yes/no), are proposed to be sufficient to localize and consecutively trigger communication at adhesion plaques in MCCNs.

The same examples that illustrate programmability may be adduced as instances with respect to the input-process-output steps. For instance, the sequence of the DNA strands, the architecture of a MCCN, the temperature or external variables in general, the phospholipids used, and the allocation of substances in a MCCN represents the inputs. Processing of these different inputs results in outputs such as a sorting process of the vesicles, a defined architecture, and the production and release of active drugs. The output may manifest itself either in a morphological configuration (e.g., defined architecture of MCCNs) or in a substance (e.g., active drug molecules). This dichotomy and the lack of a digital form we became accustomed to in the computer age is the kernel of morphological computation.

Although the abovementioned examples epitomize the notion of programmability and input-process-output, one notices the lack of a comprehensive theory of morphological computation. After 20 years of research, what makes it difficult to develop such a theory? To address this question one has to contrast the situation of 'classical' and 'morphological' computation.

Logic gates that perform Boolean logic operations in digital circuits have been implemented in hardware for a century and more recently in molecular machines that should provide a moleculator in the future (cf., the pioneering work of Credi *et al.* [34]). Like Tessla, who began to list electronic properties of vacuum tubes in 1898, and after a decade of archiving logic gates for molecular computation (see the latest list of implemented Boolean logic operations in [35]), we should start systematically investigating operators in morphological computation.

Unlike the limited number of Boolean operators used in ICT to implement digital circuits, material properties offer an almost inexhaustible number of possibilities. Nature draws on these resources and leaves the exploitation of the material properties to the genome. The fact that genomes represent a Boolean network may be traced back to the fact that material properties not are encoded per se, but to when and where materials are produced; in the given situation materials then behave according to their inherent properties.

Nature had millions of years to select materials based on their properties to decide when and where they should be expressed. The PACE project took this evolutionary aspect explicitly into account. The fact that synthetic biology dreams of the development of an 'even better mouse' shows that research believes that even nature does not conclusively exploit all technically feasible solutions. Due to the open-ended character of the exploitation of material properties, morphological computation will remain an incomplete patchwork of examples required to conscientiously list and to technically utilize material properties, as well as their coding.

1.2.3 Functionality

The description of functionality, therefore, also remains limited to the listing of examples of how material properties affect, support, or enable functionalities.

The vesicles used in drug delivery applications evolved for the last 30 years from 'plain' vesicles, with water soluble drug to new-generation drug delivery tools exposing protective polymers and targeting ligands among an almost unlimited number of modifications [36]. In contrast to these highly elaborated but technically demanding methods to extend circulation and content retention time, Boyer and Zasadzinski [37] reported a straightforward protocol to achieve the same objective. By simply encapsulating unilamellar vesicles within a second bilayer to form multicompartment "vesosomes" premature drug release, likely due to enzyme de-

gradation or protein insertion into the liposome membrane, was significantly reduced. The morphological character of a bilayer-within-a-bilayer structure alone is sufficient to increase drug retention from minutes to hours when using vesosomes. The new experimental protocol to encapsulate vesicles presented in this thesis terminates the linkage of vesicle assembly and encapsulation inherent to Boyer's and Zasadzinski's protocol.

The drug delivery system proposed in chapter 7 integrates different stimuli as input, such as the ineffective lymphatic drainage at tumor tissues and changes in pH, redox potential, temperature, magnetic field, or ultrasound at the target site [38]. The desired functionality of the encapsulated multivesicle assemblies of programmable architecture in drug delivery and personalized medicine emerges directly from the production of active drugs at the time and place where they are needed.

2 Conclusion

This thesis reveals the imperative character of the exploitation of concepts like emergence and self-organization, as well as the exploitation of material and morphological properties in implementing programmability, information processing, and teleological embedding in future man-made technology.

Despite numerous efforts in PACE and EES, a FPAC is still missing. However, by providing first instances of experimental prototypes of FPAACs, this thesis contributed significantly to the linking of ICT and LT and to the setting of a course toward completed, current, and future research efforts of the European Commission and the Swiss National Science Foundation.

Novel experimental protocols that increase procedural manageability allow for the high-throughput analyses of parameters affecting artificial vesicles, and enable the internally structuring of vesicles by artificial organelles.

Although several examples of application in fields as diverse as bioreactors, personalized healthcare, software-wetware testbeds, design of novel antiviral drugs, and membrane computing were discussed, the potential of this new LICT has not been exhausted.

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Programmable Artificial Cell Evolution (PACE) - Artificial Cells as Building Blocks of Microscopic Modular Robotic Systems. Institute of Plant Biology, University of Zurich, Switzerland (invited by Prof. Enrico Martinoia).

2007 *Vesicle Self-Assembly*. European Center for Living Technology, Ca' Minich, Venice, Italy (invited by Dr. Ruedi Füchslin).

Popular Communication

2008 *PACE: Artificial Cells as Building Blocks of Modular Robotic Systems*. Department of Informatics, University of Zurich, Switzerland (AILab 20th Anniversary).

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Posters

2010 **Hadorn M**, Boenzli E, Eggenberger Hotz P. *Does Systems Biology have to get Wet?* Workshop of the European Molecular Biology Organization (EMBO) on Systems Biology of Development. Ascona, Switzerland.

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APPENDIX 1

FUNCTIONALITY: TO ALLOW FOR ANTIVIRAL DRUG DESIGN

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Meta-Engineering in Drug Design: A Roadmap to Implement a Novel Liposome-Based Testbed

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ABSTRACT. Retroviruses cause a variety of the most serious diseases of man and animals. A new class of antiretroviral drugs, so-called 'entry inhibitors', block virus entry into the host cell. Until now, antiretroviral drug have been designed and evaluated in complex natural virus-cell systems, but the complexity impedes detailed insights into the underlying molecular mechanisms of virus-cell interaction. Therefore, we propose to engineer a novel model system which reduces the complexity of the components involved in virus-cell interaction. The proposed artificial model system will provide an *in vitro* testbed for antiretroviral drug design and validation. The system will combine the advantages of natural and artificial models by consisting of artificial liposomes equipped with a minimal cellular machinery providing nothing but the components needed for the molecular processes in virus-cell interaction. We are able to refer to data of several 'entry inhibitors' tested in a natural virus-cell system, and we already established liposome containers separated from the surrounding by a lipid membrane that enclose sugars, and amino acids. Here, we present results of encapsulating nucleotides and organic and inorganic ions. Further, we discuss how to proceed on the way towards a novel liposome-based testbed for antiretroviral drug design and validation.

KEYWORDS. Inhibitory peptides, Liposomes, Cell-free expression system, Living Technology

INTRODUCTION

Retroviruses (e.g. HIV) incorporate their genes into the host's genome and thus establish life-long infections that frequently terminate in fatal diseases (e.g. AIDS). Since xenotransplantations [1] will gain in importance, risks concerning the transfer of animal retroviruses to humans have to be assessed beforehand. Feline leukemia virus [2], a naturally occurring gammaretrovirus of domestic cats and some related small felids [3, 4], serves both as a model of the multifaceted pathogenesis of retroviruses (i.e. in tumor and AIDS research) [3] and as a testbed for the risk assessment of xenotransplantation. For most retroviral diseases, therapies are either absent or have the disadvantage of developing drug resistance and/or having high toxicity profile. Recently, a novel promising antiretroviral drug class was developed: Small synthetic peptides, termed 'entry inhibitors' or 'fusion inhibitors'. Operating very early in the viral life cycle, they interfere with binding onto the surface of the host cell, virus and host cell membrane fusion, and virus entry into the host cell [5-7]. In contrast to other antiretroviral drugs, fusion inhibitors have a low toxicity profile [8, 9].

In the development of antiretroviral therapies one attempts to exploit the retroviral vulnerability of a general structure and a simple genomic organization. The viral gene *env* [10] encodes for an

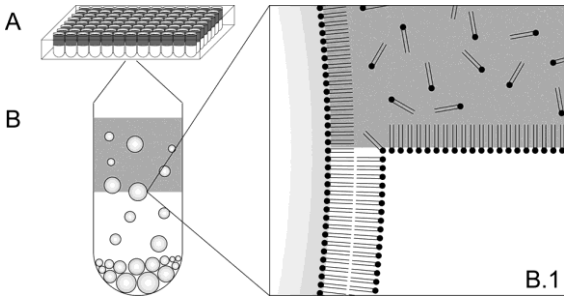


Fig. 1. Schematic representation of the parallel liposome formation. (A) Liposomes are produced in 96-well microtiter plates, providing parallel formation of up to 96 distinct liposome populations. (B) The sample is composed of two parts: water droplets (light gray) in the oil phase (dark gray), hosting nucleic acids (cp. Figure 2) or (in)organic ions (cp. Table 1) and the bottom aqueous phase (white), which finally hosts the liposomes. (B.1) Due to their amphiphilic character, phospholipids (black), solved in mineral oil, stabilize water-oil interfaces by forming two monolayers. These two monolayers form a bilayer when a water droplet, induced by centrifugation, passes the interface. Due to both the density difference of the *inter*- and *intra*-liposomal fluid and the geometry of the microplate bottom, vesicles pelletize in the centre of the well (cp. B).

envelope protein inducing viral cell entry: Interaction of the envelope protein and a specific host cell surface receptor induces a fusion of the virus and host cell membranes [11]. Virus subgroups differ in the characteristics of the envelope protein resulting in differences in cell targeting and specific disease capacity [12-16]. The virus host cell receptors for all FeLV subgroups have been defined recently [13-15, 17-24]. As point of vantage the interaction of virus envelope protein and specific host cell receptors has been studied extensively in *in vitro* systems that use natural cells [25]. Since in natural cells a tremendous number of various processes take place simultaneously, such systems often fail to elucidate elementary molecular mechanisms of the system. Thus, in the last years, serious efforts were made to replace the natural virus-cell system by an artificial virus-liposome model. Liposomes feature an aqueous compartment partitioned off the surrounding by an impermeable lipid membrane. The simplicity in structure reduces the complexity of the system [26-28]. On the other hand, the complete absence of cellular components in the artificial virus-liposome systems has unfavorable effects on validity and comparability with the virus-cell and the biological (*in vivo*) system. Therefore, we propose to engineer a novel virus-liposome model system which is simple to understand but complex enough to draw conclusions on the natural system. Only recently, wet-laboratory approaches in engineering and meta-engineering spanning a wide variety of research disciplines have been unified in the concept of Living Technology [29]. The growing field of Living Technology is likely to gain in importance in new engineering disciplines with multiple applications in the medical, material, information, energy, and environmental sciences [30].

In this paper, we characterize the basic specifications, summarize what has been achieved so far, present our current results, and discuss how to realize the missing components to engineer the novel virus-liposome model system that will provide a testbed for antiretroviral drug design and validation. The system will combine the advantages of the virus-cell and the virus-liposome models by consisting of liposomes equipped with a cellular machinery composed of a minimal number of components and providing nothing but the molecular processes of the virus-cell interaction.

For the implementation of the testbed, the following components need to be realized: (i) A cell-like container (ii) encapsulating the minimal cellular machinery providing protein synthesis and (iii) data of 'entry inhibitors' collected in a natural system.

(i) A cell-like container: Liposomes are the most studied systems among biomimetic structures [31] and have frequently been used as models for living cellular structures [32], since they make an ideal tool for investigating enclosed systems containing ongoing biochemical processes, including the replication of RNAs [33], the PCR [34], and polypeptide synthesis [35]. Over the last decades, several *in vitro* liposome formation and analytical characterization procedures were developed (for a review see [36]).

(ii) Encapsulation of the minimal cellular machinery: Commercially available cell-free expression systems

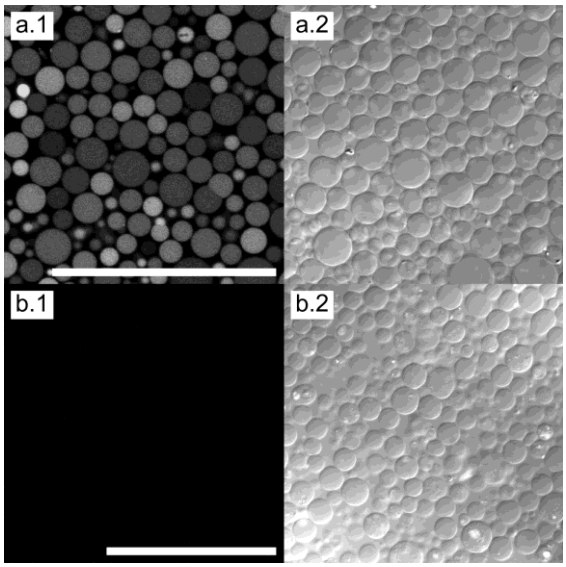


Fig. 2. *Encapsulation of nucleic acids.* (a.1, b.1) Confocal laser scanning microscope and (a.2, b.2) differential interference contrast micrographs of two liposome populations. Only liposome population (a) incorporates fluorescently labeled DNA strands. Liposome population (b) does not hold DNA strands and is used as control. Scale bar represents 100μm.

provide the minimal cellular machinery to synthesize proteins in vitro [37, 38]. Usually cell-free expression systems that originate from wheat germ or *Escherichia coli* (*E. coli*) are used. Though synthesis of soluble proteins in liposomes is well established [32, 39-42], expression of membrane-associated proteins is restricted to spontaneously inserting hemolytic proteins that do not rely on cellular machinery providing appropriate protein localization [43, 44]. Only recently, the appropriate protein localization of integral membrane proteins was reported for a novel expression system consisting of a fusion between *E. coli* inner membrane protein (GlpF) and eukaryotic integral membrane proteins [45]. This system allows for the synthesis of eukaryotic integral membrane proteins in *E. coli*.

(iii) Data of 'entry inhibitors': Inhibitory peptides were already designed and analyzed *in vitro* in a natural virus-cell system [46]. These results providing data to validate the quality of a natural system (virus-cell) are necessary to verify and validate an artificial virus-liposome testbed.

By introducing microtiter plates in liposome formation and increasing the versatility [47, 48] of an established liposome formation procedure [44, 49, 50], we provide parallel and high-throughput analyses [47]. In addition, we implemented and evaluated a DNA-mediated self-assembly procedure to generate multi-compartment aggregates of programmable and predefined composition [47, 51] and discussed their application in personalized medicine [52].

Introducing asymmetry in the *inter-* and *intra*-liposomal fluid results both in an independent composition control of the inner and outer medium, and in an increased liposome manageability [47, 48, 52]. Broadly speaking, natural cells contain sugars that provide energy for cells, lipids that build up the cell membranes, amino acids that are the subunits of proteins, and nucleotides that code for the development and functioning of all known living organisms. Artificial liposome membranes are made of lipids as well. From the remaining three major families of small organic molecules we already tested the encapsulation of sugars [47, 48] and amino acids. Here, we therefore analyzed liposome formation and liposome stability in dependence of incorporation of nucleic acids (DNA strands). Moreover, organic and inorganic ions are essential to all cellular processes. Thus, to set up artificial liposomes hosting the cellular machinery needed to express proteins, one has to provide not only the genetic blueprint (DNA strands) but also a cell-like composition of the *intra*-liposomal fluid. We therefore analyzed liposome formation and liposome stability in dependence of incorporation of both organic and inorganic ions in this study.

By engineering a simple but more cell-like virus-liposome model system, we expect to provide a high-throughput testbed for the design and validation of novel antiretroviral drugs.

MATERIALS AND METHODS

The established liposome formation protocol [49] was technically modified as follows: introduction of (i) 96-well microtiter plates U96 to increase procedural manageability in laboratory experimentation and (ii) a density difference between *inter-* and *intra*-liposomal solution to induce liposome pelletization (Figure 1). Solutions of the liposome lumen and

Table 1. Relative liposome yield in dependence of (in)organic sodium salts. The liposome yield is expressed as a percentage of the control (*intra*-liposomal fluid without the addition of salt).

	50 millimolar	10 millimolar
Positive control	100.000 ± 1.779	100.000 ± 3.581
Negative control, Sodium dihydrogenphosphate, $H_2NaOP \bullet 2\ H_2O$,	0	0
Sodium formate, $CHNaO_2$	0	97.401 ± 5.100
Sodium N-lauroylsarcosinate, $C_{15}H_{23}NNaO_3$	0	105.924 ± 3.910
Sodium acetate trihydrate, $CH_3COONa \cdot 3\ H_2O$	88.893 ± 6.350	100.793 ± 8.964
Sodium hydrogen carbonate, $NaHCO_3$	97.185 ± 1.754	105.291 ± 3.899
Sodium carbonate, Na_2CO_3	99.426 ± 6.221	103.075 ± 3.859

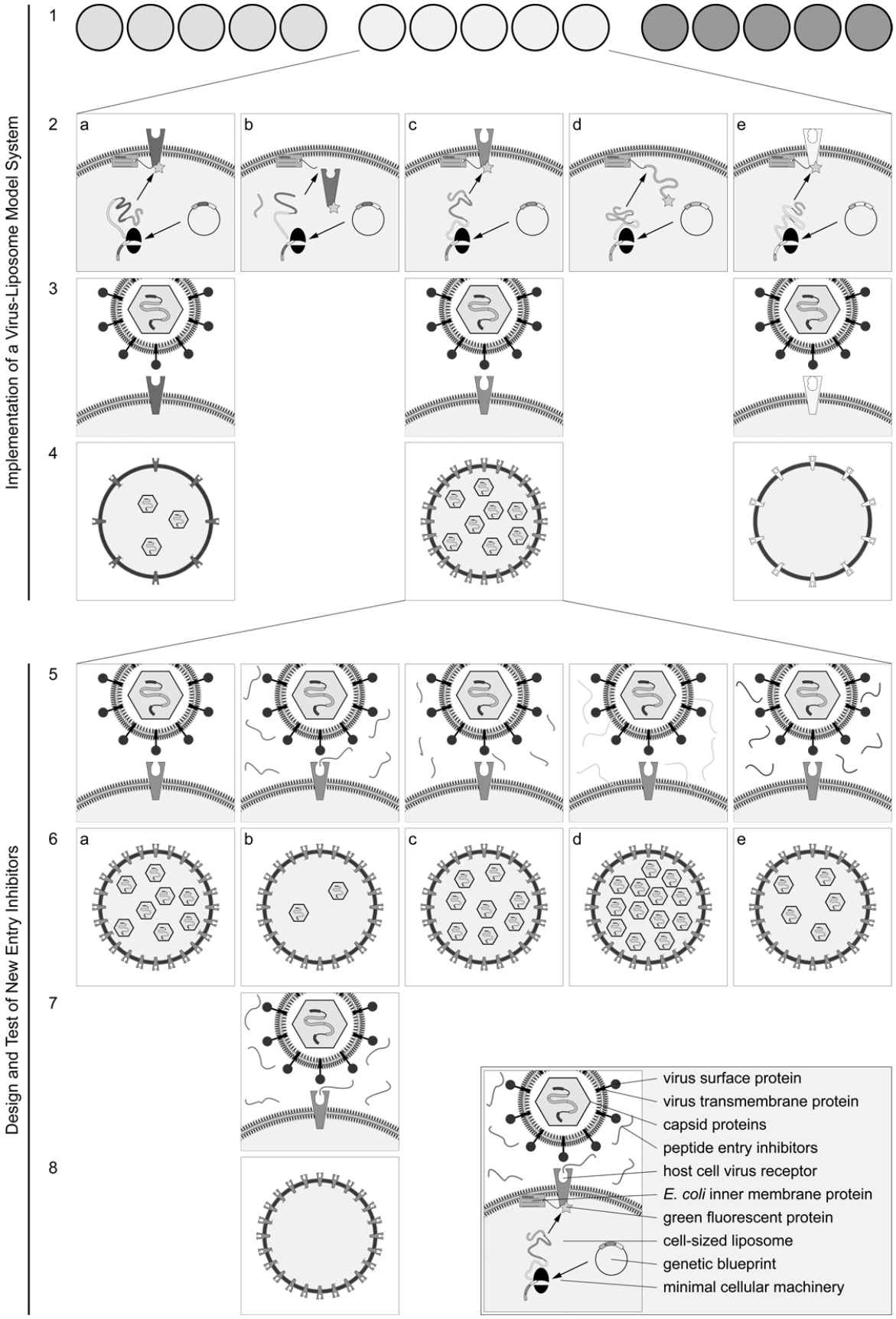
the surrounding medium were equal in osmolarity but differed in the degree of polymerization of dissolved saccharides (*intra*-liposomal: disaccharides, *inter*-liposomal: monosaccharides) providing density differences between the lumen and the environment. The sample was composed of two parts: (i) an oil phase hosting water droplets with sucrose (disaccharide) and either fluorescently labeled (Alexa Fluor 488) DNA strands (10 micromolar) or (in)organic ions (for a list of tested ions and concentrations, see captions of Table 1) as additives and (ii) the bottom aqueous phase, which finally receives the liposomes. Due to their amphiphilic character, phospholipids (dissolved in mineral oil) stabilize water-oil interfaces by forming monolayers. Two monolayers form a bilayer when a water droplet, induced by centrifugation, passes the interface. Liposome membranes were exclusively made of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.

Due to both the density difference of the *inter*- and *intra*-liposomal fluid and the geometry of the microplate bottom, liposomes pelletize in the centre of the well. Liposome formation was performed in duplicates. Length of circumference of the liposome pellet is used as a measure of liposome yield (cp. methodology in [47]). The liposome yield was compared to the control (without addition of ions) providing values of relative liposome yield. Light-microscopy was performed using a Wild M40 inverted microscope equipped with a MikoOkular microscope camera. All camera settings were identical for the recordings. Confocal laser scanning microscopy was performed using an inverted Leica Confocal DMR IRE2 SP2 confocal laser scanning microscope.

RESULTS AND DISCUSSION

Liposomes were found to sediment and hence to be easily available for inverse microscopy (Figure 2). The fluorescence signal was found exclusively in the lumen of the liposomes and only if fluorescently labeled DNA strands were present during liposome formation (Figure 2.a.1). Autofluorescence was absent (Figure 2.b.1). Thus, one can conclude that nucleic acids are incorporated efficiently into the

Fig. 3. (opposite). Schematic representation of the methodological procedure to engineer a novel virus-liposome model system that provides an effective testbed for antiretroviral drug design and validation. (1) Three different commercially available cell-free expression systems are depicted, hereafter details are provided for one of these. (2) In each transcription-translation cell-free expression system a distinct host cell protein is expressed that is assumed to enable virus cell entry. Concerning protein expression and localization different results are conceivable: (a, c, e) gene expression, size, native conformation and the membrane localization of the fusion proteins are adequate; (b) mislocalization or (d) misfolding of the host cell receptor. (3) The number of virus-liposome model systems is reduced – only 'convenient' (see text) combinations of liposome stability, cell-free expression system, fusion protein expression and localization are further used and incubated with native virus. (4) The virus load differs in dependence of host cell surface receptor density and its accuracy in respect to the virus envelope protein. The most promising virus-liposome model system is selected for the further procedure. (5) Peptide entry inhibitors that differ in length and/or sequence either decrease (6.b, 6.e), not affect (6.c), or increase (6.d) the virus load compared to the control (6.a). (7) The inhibitory potential of the most promising antiretroviral peptide is increased using an iterative in silico optimization procedure, finally resulting in a potent antiretroviral drug (8).


Implementation of a Virus-Liposome Model System
Design and Test of New Entry Inhibitors
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2
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e
7
8
virus surface protein
virus transmembrane protein
capsid proteins
peptide entry inhibitors
host cell virus receptor
E. coli inner membrane protein
green fluorescent protein
cell-sized liposome
genetic blueprint
minimal cellular machinery

Fig. 3.

liposomal lumen if present during liposome formation.

The (in)organic molecular sodium salts tested (Table 1) only differed in the anionic component. The relative liposome yield differed in dependence of the concentration of the sodium salt. At a concentration of 10 millimolar the liposome yield is either the same or higher than in the control experiment except for sodium dihydrogenphosphate. Sodium dihydrogenphosphate was chosen as a negative control, due to its capacity to prevent oil separation. Sodium phosphates are therefore used as emulsifiers and detergents. Thus, as expected liposome formation is inhibited completely in the presence of sodium dihydrogenphosphate. At 50 millimolar sodium formate and sodium N-lauroylsarcosinate prevent liposome formation as well. Both are amphiphilic, therefore probably destabilizing the phospholipid monolayers needed for the liposome production. The negative effect on liposome formation observed for most halogen sodium salts (e.g. sodium chloride; data not shown), is absent for most of the (in)organic molecular sodium salts tested in this study. (In)Organic molecular sodium salts may therefore provide viable alternatives to halogen sodium salts when it comes up to providing cell-like composition of the *intra*-liposomal fluid.

We implemented containers partitioned off the surrounding by a lipid membrane, we were able to enclose sugars, amino acids, nucleotides, organic and inorganic ions, and we will be able to refer to data of several 'entry inhibitors' already tested in an established virus-cell model system. In the remaining paragraphs we point out how to proceed on the road towards a novel virus-liposome model system that may provide an effective testbed for antiretroviral drug design and validation (Figure 3).

Different cell-free expression systems are commercially available. Encapsulation of the cell-free expression systems may be performed using established procedures [44]. The genetic blueprint to produce host cell virus receptor proteins is composed of three different parts (Figure 3.2): i) *E. coli* inner membrane protein GlpF [45], providing appropriate protein localization of integral membrane host cell virus receptor proteins, ii) one of the host cell virus receptor candidates that are assumed to enable virus-liposome interaction and membrane fusion, and iii) a green fluorescent protein (GFP) providing information about the protein localization. The three genetic components may be cloned and expressed in cell-free expression systems available.

Routine procedures for cloning [53] are available and the sequence of the constructs can be amplified by polymerase chain reaction (PCR) and inserted into the vector pI VEX 2.3d (cp. [54]). All constructs may be verified by sequencing and gene expression, size, native conformation, and the proper membrane localization of the proteins may be analyzed for each combination of cell-free expression system and fusion protein by using RT(reverse transcriptase)-PCR, western blotting, fluorescence and confocal microscopy, and ELISA (enzyme-linked-immunosorbent assay) techniques. The aim should be to establish 'viable' combinations of liposome stability, cell-free expression system, fusion protein expression and localization.

Having appropriately equipped liposomes, the capacity of FeLV to enter the liposome mediated by the host cell receptor on the surface will be analyzed by co-incubating liposomes and virus (Figure 3.3), removing of excess virus, lysis of liposomes, and quantification of virus load using RT-PCR (Figure 3.4). Precautions have to be taken to prevent that the selection of the most promising virus-liposome model system (Figure 4.4) not only depends on the receptor surface density (as implied in Figure 4) but also on the quality of attachment of receptor and virus envelope protein (cp. geometrical match of receptor and virus envelope protein is better for the virus-liposome model system (a) of Figure 4.4 but the larger number of receptors is sufficient that the virus-liposome model system (c) outperforms (a) that would actually be better suited). To prevent such a problem, virus load will have to be correlated with the receptor surface density quantified for example by using the GFP-fluorescence signal.

To review the appropriateness of the novel model system liposomes and virus are co-incubated either in the presence or absence of peptide entry inhibitors (Figure 3.5) whose antiretroviral activity have

already been tested [46]. The liposome virus load is quantified (Figure 3.6) and compared to the virus-cell system resulting in a qualitative comparison of the two model systems. Based on these data, a genetic algorithm to evolutionary design experiments in wetware established by Poli and coworkers [55] will be used to design new antiviral peptides, tested, and iteratively optimized (Figure 3.7), resulting in potent peptide entry inhibitors (Figure 3.8).

CONCLUSIONS

In previous work, we implemented cell-like containers separated from the surrounding by a lipid membrane that enclose sugars and amino acids. In the present study, we enlarged the range of substances enclosed by nucleotides and organic and inorganic ions. Thus, all substances are incorporated that are required to implement a basic metabolism within liposomes. The simplicity of these elementary cell-like containers providing a basic metabolism may be exploited in the design of an effective liposome-based testbed for antiretroviral drug design and validation. Since the inhibitory potentials of some instances of a new class of antiretroviral drugs are already tested using common *in vitro* systems, the effectiveness of the new liposome-based testbed in validation of antiretroviral drugs may be evaluated. The new liposome-based testbed potentially offers high-throughput analyses of antiviral drugs and may optimize or open up bottlenecks inherent to current technologies applied in drug design.

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